Functional Characterization of 14-3-3 Proteins and Exonuclease1 at Stalled Replication Forks

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Zürich, 2010

-to my parents-

-fir méng Elteren-

Table of contents

SUMMARY

DNA replication and DNA repair are two tightly linked processes since, on the one hand, errors occurring during replication in germline cells might introduce mutations that are handed on to the next generation and, on the other hand, unrepaired DNA structures might cause replication blocks that threaten genome integrity. DNA and replication fork integrity are monitored by checkpoint-mediated phosphorylation events that trigger repair pathways.

Exonuclease 1 (Exo1) processes stalled replication forks in checkpoint-defective yeast cells. While studying Exo1 and its regulation by phosphorylation and other post-translational modifications, we isolated an interesting group of novel *in vivo* interaction partners in yeast and mammalian cells, namely the 14-3-3 proteins. 14-3-3's are able to bind phosphorylated proteins and, in addition to their well-known ability to act as adaptors that integrate signals from different pathways, they were shown to play an undefined role under DNA replication stress. The finding that they interact with Exo1 led us to formulate the hypothesis that the 14-3-3/Exo1 complex might have a functional role at replication forks and encouraged us to investigate this possibility.

Using DNA bi-dimensional electrophoresis, we could show that yeast 14-3-3's promote fork progression under limiting nucleotide concentrations. 14-3-3-deficient cells fail to induce Mec1-dependent Exo1 hyper-phosphorylation and accumulate Exo1-dependent ssDNA gaps at stalled forks, as revealed by electron microscopy. This leads to persistent checkpoint activation and exacerbated recovery defects. Interestingly the fork progression defect in 14-3-3 cells cannot be rescued by Exo1 deletion and the recovery defect is only partially rescued by Exo1 deletion, suggesting that additionally to Exo1, 14-3-3 proteins might regulate the phosphorylation of other yet unknown targets in response to replication fork stalling.

Based on this evidence, we propose that 14-3-3 proteins assist checkpoint-mediated phosphorylation of Exo1 and additional unknown targets, promoting fork progression, stability and restart in response to DNA replication stress.

ZUSAMMENFASSUNG

Die Replikation und die Reparatur der DNS sind zwei eng verknüpfte Prozesse. Auf der einen Seite können Fehler während der Replikation von Keimbahnzellen Mutationen hervorrufen welche an die nächste Generation weitervererbt werden. Andererseits können abnormale DNS Strukturen die Replikationsgabeln blockieren, was die Integrität des Genoms gefährden kann. Die Integrität der DNS und der Replikationsgabeln wird von Checkpoint-Kinasen überwacht, welche Reparatur Signalkaskaden auslösen können.

In Hefezellen, welche einen Checkpoint Defekt haben, prozessiert Exo1 arretierte Replikationsgabeln. Während unserer Studie im Zusammenhang mit der Regulierung von Exo1 durch Phosphorylierung und andere post-translationalen Modifikationen, haben wir eine interessante Gruppe von bisher unbekannten *in vivo* Interaktionspartnern gefunden, die 14-3-3 Proteine. Diese sind in der Lage phosphorylierte Proteine zu binden und, zusätzlich zu ihren hinreichend bekannten Fähigkeiten als Adaptoren, welche Signale von verschiedenen Signalkaskaden integrieren, konnte gezeigt werden, daß sie eine bisher nicht genauer definierte Rolle während des DNS Replikationsstresses spielen. Die Isolation von 14-3-3 als Interaktionspartner von Exo1, hat uns ermuntert die von uns postulierte Hypothese zu untersuchen, welche besagt, daß der 14-3-3/Exo1 Komplex eine funktionelle Rolle an Replikationsgabeln spielt.

Wir konnten anschließend dank der bi-dimensionalen Elektrophorese zeigen, daß, wenn die Nukleotidkonzentrationen limitierend sind, 14-3-3 Proteine die Progression der Replikationsgabeln fördern. In 14-3-3 defizienten Zellen wird die Mec1 abhängige Exo1 Hyperphosphorylierung nur mangelhaft induziert. Wie wir anhand der applizierten Elektronenmikroskopie zeigen konnten, akkumulieren die 14-3-3 defizienten Zellen Exo1 abhängige ssDNS Lücken an den arretierten Replikationsgabeln. Diese Lücken führen zu einer persistenten Checkpoint Aktivierung und Defekten bei der Erholung vom Replikationsblock. Interessanterweise kann der progressions-Defekt der Replikationsgabeln nicht durch die Deletion von *EXO1* verhindert werden. Zusätzlich wird der Defekt während der Erholung vom Replikationsblock nur partiell durch die Deletion von *EXO1* verhindert. Dies suggeriert, daß zusätzlich

zu Exo1, 14-3-3 noch weitere bisher unbekannte Zielproteine über deren Phophorylierungs-Status reguliert, wenn es zu einer Blockierung der Replikationsgabeln kommt.

Gestützt auf diese Resultate postulieren wir, daß 14-3-3 Proteine Checkpoint vermittelte Phosphorylierungen auf Exo1 und auf zusätzliche, bisher unbekannte Ziele, regulieren, wodurch sie die Progression, die Stabilität und den Neustart der Replikationsgabeln fördern.

1. INTRODUCTION

1.1. DNA replication and repair

1.1.1. General features of chromosome replication

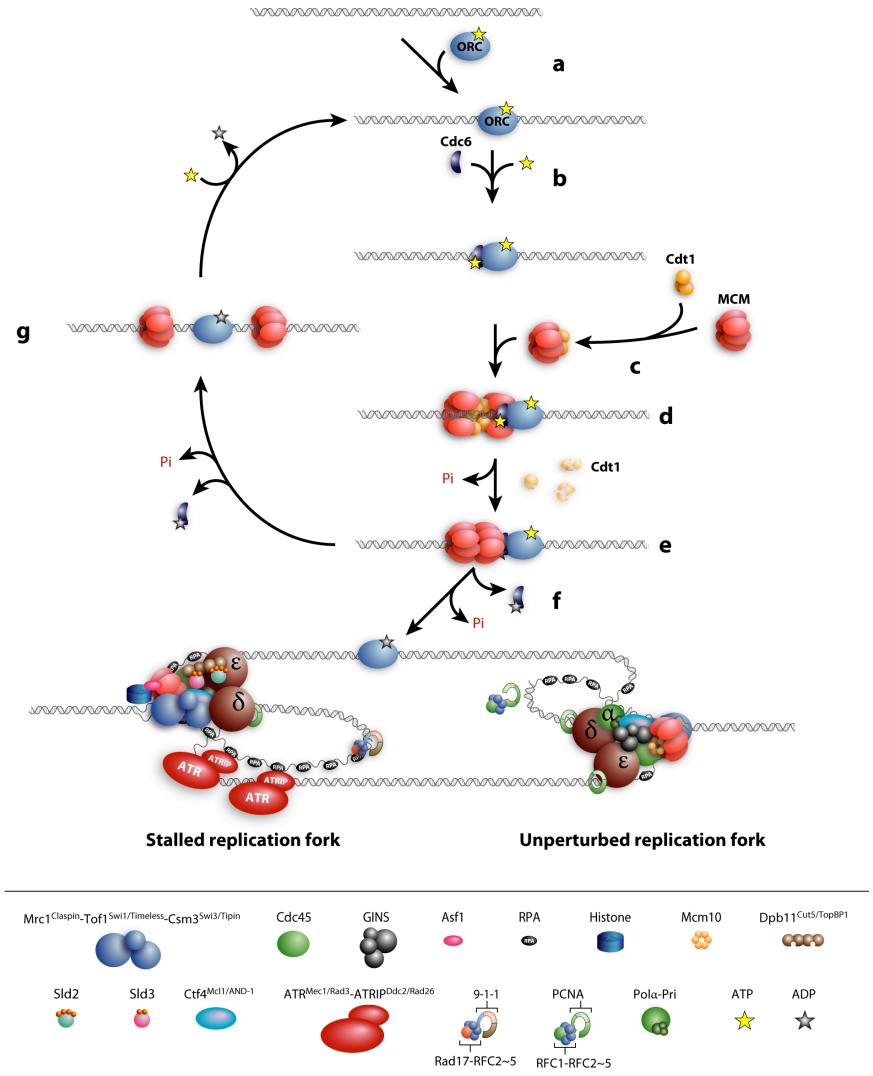
The groundwork for understanding of chromosomal replication was laid by Crick and Watson and their famous DNA duplex structure model, which suggested that the two new DNA strands are copied from the old strand thus allowing exact copying of genetic information. The two old strands reassemble with two newly synthesized daughter strands in a semiconservative manner. Most eukaryotes and prokaryotes carry out DNA replication bidirectionally from a given start point, often referred to as origin of replication. Most circular DNA molecules present in bacteria and viruses have only one origin of replication and the two growing forks merge on the opposite side of the circle to complete replication. Multiple origins are present in the long linear chromosomal DNA of eukaryotes. Growing forks from neighboring origins advance until they meet and likewise complete the replication of one so-called replicon. For example the human genome contains about $3*10^9$ base pairs and it takes roughly 8 hours to complete DNA replication of the approximately 10.000 to 100.000 replicons. DNA replication is tightly monitored to ensure accurate replication of the chromosomes just once per cell cycle and completion of the replication before the onset of mitosis. The sequence of events and the components of the DNA replication machinery are highly conserved throughout the entire eukaryotic world (Brush and Kelly, 1995).

1.1.2. Initiation of chromosome replication

Whether, when and where DNA replication begins is tightly controlled during the initiation step of replication, the origin firing. Replication origins of all species share the following properties: Their DNA segments contain multiple short repeated sequences. These sequences are recognized by multimer origin binding proteins that play a key role in assembling the replisome. Origins of replication contain AT rich sequences that facilitate unwinding of duplex DNA.

Some organisms, like the budding yeast *Saccharomyces cerevisiae*, have clear replicator sequences called autonomously replicating sequence (ARS), whereas many other organisms have less strict DNA sequence requirements for the initiation events. The yeast *S. cerevisiae* is especially useful when it comes to studying origins of replication and DNA replication itself. In this model organism 400 origins of replication exist on the 17 Chromosomes and a dozen of these have been characterized in detail. The yeast origin sequences (ARS), when introduced into a plasmid, confer the ability to replicate in yeast.

Binding of the origin recognition complex (ORC) to the origins allows the pre-replicative complex (preRC) to associate on the origins in G1, and the recruitment of additional replication factors like cell division control protein 6 (Cdc6), the chromatin licensing and DNA replication factor 1 (Cdt1) and the minichromosome maintenance (MCM) helicase complex to these sites before the onset of DNA replication. Chromatin structures are important factors not only for transcription but also for origin selection and they seem to affect the efficiency of preRC assembly, which may indirectly alter the initiation frequency at particular loci, but they are probably not essential for origin firing. At each fired origin, two sister replication forks emerge and move away from each other and this causes an inhibition of the preRC in order to prevent re-replication (Masai et al. 2010).



Masai H, et al. 2010.
Annu. Rev. Biochem. 79:89–130

Fig.1 Model of a stalled versus unperturbed replication fork

Modified after:

Masai H. et al.

1.1.3. The DNA replication machinery

DNA replication is tightly monitored to ensure accurate replication of the chromosomes just once per cell cycle and completion of the replication before the onset of mitosis. The sequence of events and the components of the DNA replication machinery are highly conserved throughout the entire eukaryotic world. After initiation of DNA replication in the S phase, the MCM complex moves away from replication origins as part of the DNA replication fork machinery. The MCM2~7, a ring-shaped hexameric complex, is the

replicative DNA helicase. This DNA unwinding helicase prepares the ground for the polymerases by melting and unwinding the DNA template and likewise removing the torsional stress ahead of the replication fork. Since a DNA polymerase can only extend an existing DNA strand paired with the parental strand, a short fragment of DNA or RNA, called primer, must be created and paired with the template strand before a DNA polymerase can synthesize a new daughter strand. A primase tightly associated with polymerase α (pol α) synthesizes a primer that serves as starting point for the associated pol α. On the leading strand, binding of the ring clamp PCNA on the primer-template terminus displaces pol α. The association of polymerase δ (pol δ) with PCNA stimulates its processivity, so that it can synthesize the remainder of the leading strand. DNA replication is believed to be continuous on the leading strand in contrast to the lagging strand where replication occurs in opposite direction of the replication forks. The lagging strand synthesis is carried out by the combined action of primase and pol α leading to the formation of so-called Okazaki fragments. The replication factor C (RFC) enhances the activity of pol α and finally a ligase is needed to close the remaining nicks on the lagging strand (Hübscher, 2009).

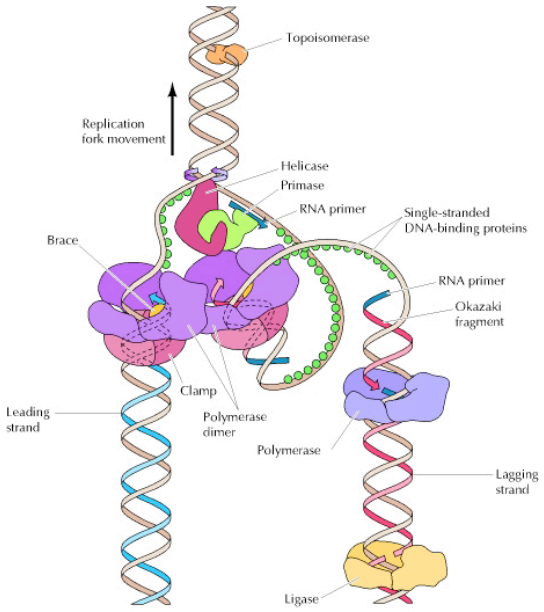


Fig.2 Schematic model of leading and lagging strand synthesis

Modified after:

Geoffrey M Cooper

Since lagging strand synthesis occurs discontinuously and in opposite direction than replication fork progression, it cannot be replicated in its entirety. The end replication problem would lead to shortening of this strand at each cell cycle and therefore, the need for a special chromosomal region, the telomeres, is apparent. In human cells, the sequence of the telomeres consists of an AGGGTT repeat and a specialized enzyme, the telomerase prevents progressive shortening by adding this repeat to the ends of DNA molecules. The catalytic activity of this unusual enzyme polymerizes deoxyribonucleotides directed by its RNA template as well as the RNA template itself.

Finally a complex of telomeric proteins, the Shelterin complex, consisting of TRF1, TRF2, Rap1, Tin2, Pot1 and TPP1, helps to protect the free DNA ends. This protective complex prevents the cells from recognizing this structure as a DNA double strand break (DSB), that otherwise would lead to a checkpoint activation and recruitment of DSB repair factors (Blasco, 2007).

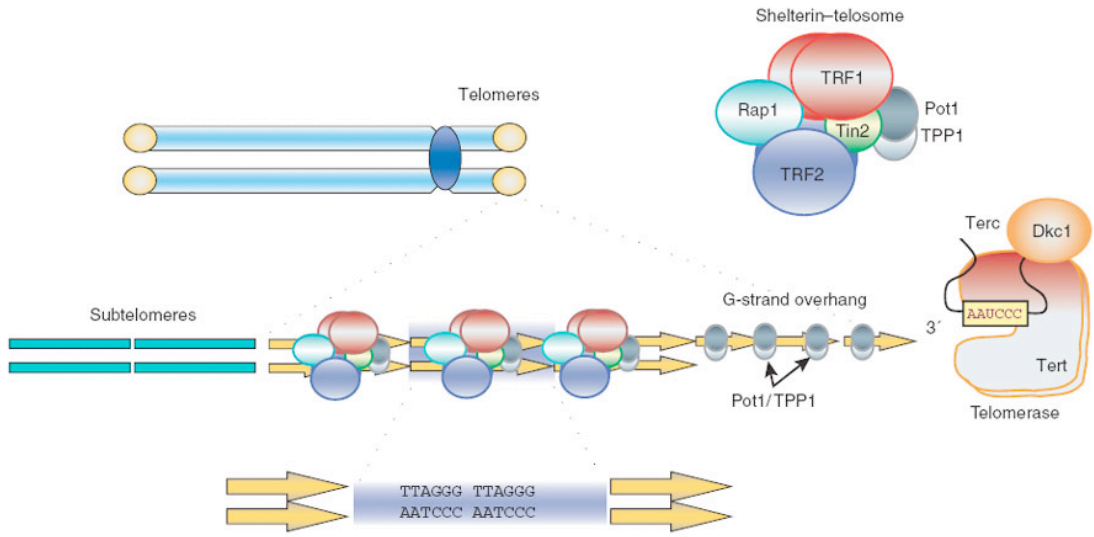


Fig.3 Schematic representation of Telomeres

Modified after:

Maria A Blasco

Nature Chemical Biology 3, 640 - 649 (2007)

1.1.4. DNA damage associated replication and repair

DNA lesions generated by exogenous agents or endogenous processes are a constant threat for the genome. Such lesions can cause stalling and

collapse of the replication fork and lead to chromosome lesions, mutations, genome rearrangements and eventually cancer (Hickson, 2003). To prevent this, a replication checkpoint has evolved as surveillance mechanism to control components of the replisome (Muzi-Falconi et al., 2003) and to allow coordinating replication with cell cycle progression and DNA repair. Besides the environmental factors such as radiation and mutagenic chemicals, copying errors are occasionally introduced by polymerases during DNA replication. The fidelity of DNA replication is being maintained by several distinct mechanisms, the first being the proofreading activity of the polymerases. If an incorrect base has been incorporated, this will cause pausing of the polymerase and its intrinsic exonucleolytic activity will then excise the misincorporated base and resume replication. Another mechanism that allows the removal of misincorporated bases that have not been detected and removed by the polymerase itself is mismatch repair. The mismatch repair machinery consisting of MutSα (Msh2/Msh6), primarily involved in base substitution and small loop mismatch repair, MutSβ (Msh2/Msh3), involved in small loop repair, in addition to large loop repair and MutLα (Mlh1/Pms2), is a strand specific system for recognizing and repairing misincorporated bases that can arise during DNA replication, for example. The hemimethylation pattern of newly synthesized daughter strands allows the mismatch repair system to excise the wrong incorporated base from the correct strand. Exo1 is the exonuclease that, upon recognition of the mismatch, excises the concerned region on the DNA in a 5'-3' orientation. The different combinations of the MutS and MutL complexes confer additional repair features such as removing erroneous insertions or deletions, to this multifaceted system (Jiricny, 2006).

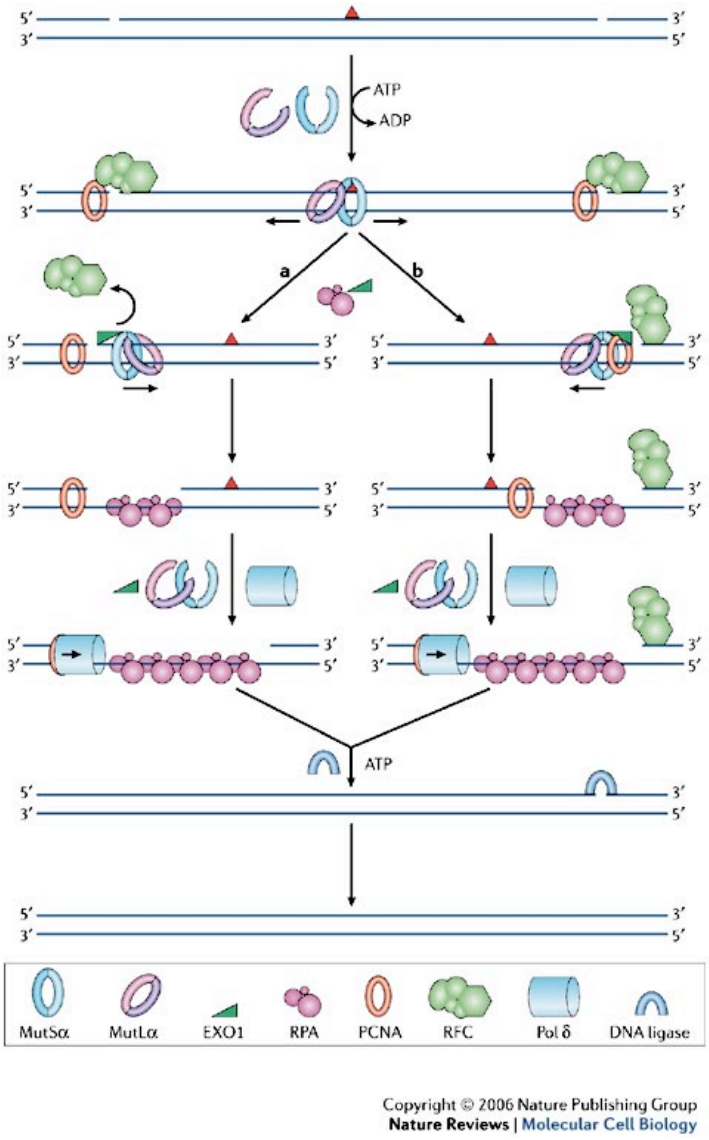


Fig.4 The mismatch repair machinery

Modified after:

Josef Jiricny

Nature Reviews Molecular Cell Biology 7, 335-346 (May 2006)

DNA damages such as bulky adducts or crosslinks, that typically arise as a consequence of UV irradiation or chemical exposure, cause stalling of the replication fork by physically blocking the polymerase.

Some of the lesions that prevent the progression of the processive polymerases can be overcome by other damage specific polymerases. A growing class of DNA polymerases, numbered ζ to ϰ, seem to be devoted specifically to overcoming damage-induced replicational stress. These special polymerases take over temporarily from the blocked replicative DNA

polymerase, polδ and pol ε, and possibly from polα. These polymerases have more flexible base-pairing properties permitting translesion synthesis (TLS), with each polymerase conferring special properties to overcome each category of injury (Maga and Hübscher, 2008).

Nucleotide excision repair (NER) is an important mechanism by which the cell can remove the vast majority of UV-induced DNA damage such as thymine dimers and 6-4-photoproducts. Xeroderma pigmentosum and Cockayne's syndrome are two severe human diseases that result from in-born genetic mutations of NER proteins. The NER enzymes recognize bulky distortions of the DNA double helix that leads to the removal of the lesion carrying short single-stranded DNA segment, likewise creating a single-strand DNA gap, which is subsequently filled in by an DNA polymerase, which uses the undamaged strand as a template (Hoeijmakers, 2009).

The cellular mechanisms that are primarily responsible for removing small, non-helix-distorting base lesions such as oxidized bases, alkylated bases and deaminated bases from the genome, is called base excision repair (BER). First, a Glycosylase removes the damaged or inappropriate bases creating so-called abasic sites (AP sites), and these are then cleaved by an AP endonuclease. Either short-patch BER can then process the generated single-strand break, where a single nucleotide is replaced by combined action of a lyase and a polymerase or long-patch BER, where 2-10 new nucleotides are synthesized by a polymerase and the displaced strand is removed by a flap endonuclease before the remaining nick is closed by a ligase.

X-rays, chemicals or replication of single-strand breaks (SSBs) and presumably during repair of interstrand crosslinks, DSBs can be induced. These are particularly dangerous lesions, since free DNA ends can cause chromosomal rearrangements and translocations that might lead to genome instability. DSBs are also problematic during mitosis as intact chromosomes are required for proper chromosome segregation during cell division (Hoeijmakers, 2001).

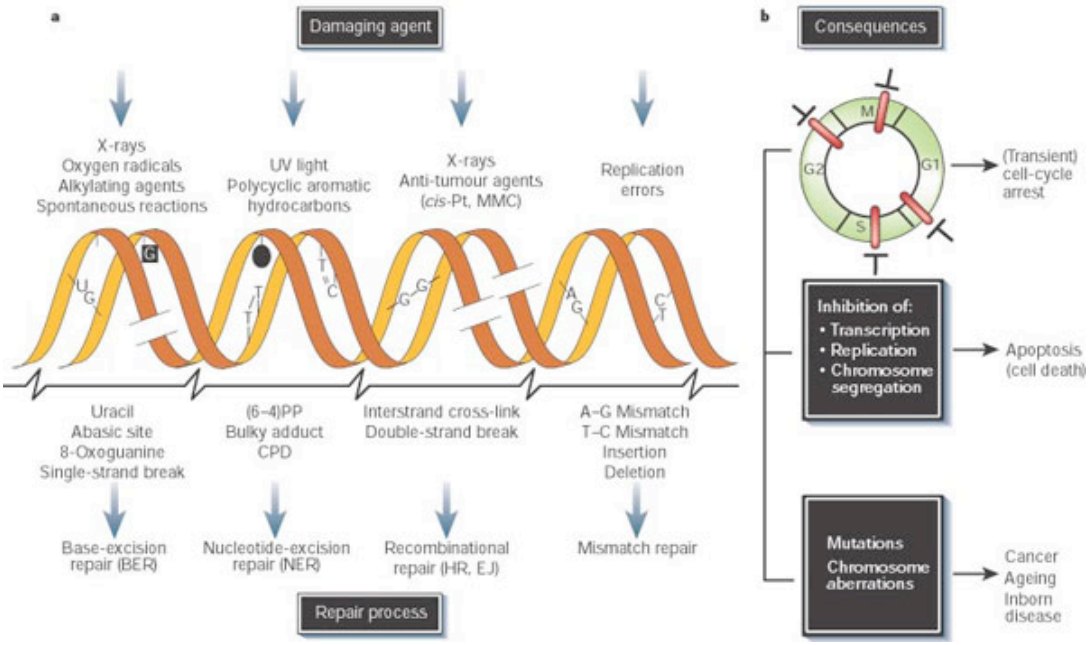


Fig.5 DNA damage and DNA repair pathways

Modified after:

Jan H. J. Hoeijemakers

Nature 411, 366-374 (17 May 2001)

Two different mechanisms address DSBs according to the cell cycle phase. During S and G2 phase of the cell cycle homologous recombination is the main pathway to repair these lesions and it is probably favoured because of the presence of the newly replicated sister chromatids, which provide a second copy of the sequence that can serve as template. During G1 phase of the cell cycle, when a second copy is not available, the error prone non-homologous end joining (NHEJ) is the main pathway to address DSBs (Downs et al. 2007)

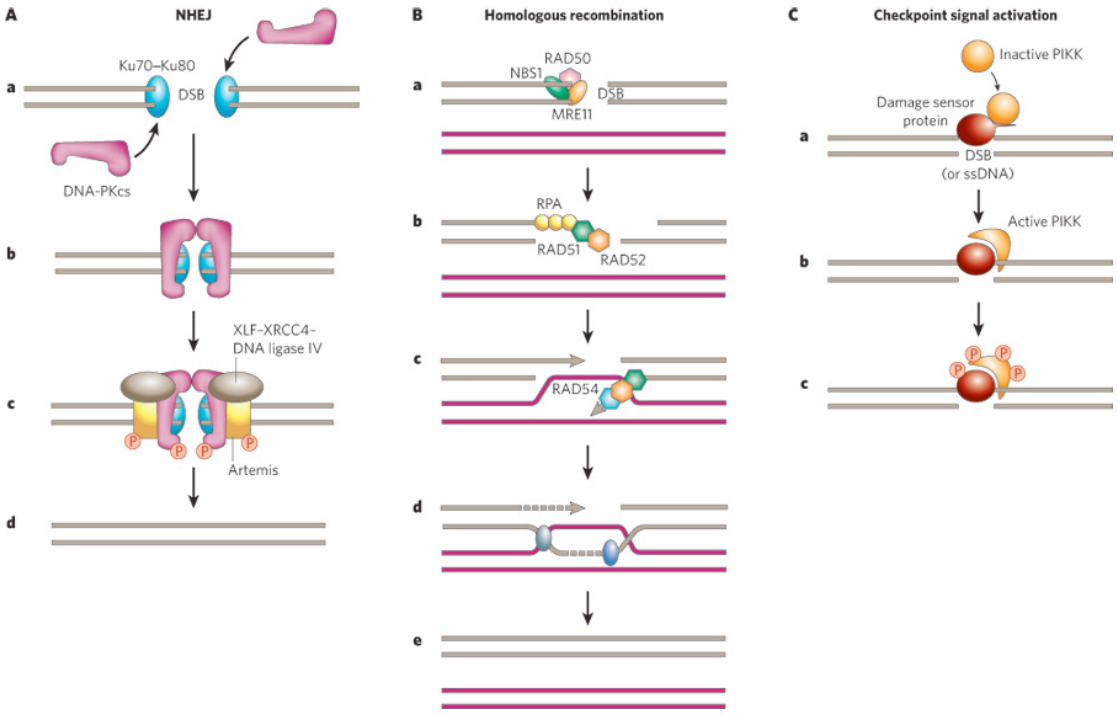


Fig.6 DSB repair by NHEJ and homologous recombination

Modified after:

Jessica A. Downs, Michel C. Nussenzweig & André Nussenzweig

Nature 447, 951-958(21 June 2007)

1.2. DNA damage response (DDR)

1.2.1. General features of the DDR

There are various, mostly negative outcomes of DNA damage. Acute consequences arise from disturbed DNA metabolism, triggering cell-cycle arrest or cell death. Long- term effects result from irreversible mutations contributing to oncogenesis.

To counteract threats posed by DNA damage, cells have evolved mechanisms, collectively termed the DNA-damage response, in order to detect DNA lesions, signal their presence and promote their repair. Defects in these mechanisms generally render cells more sensitive towards DNA-damaging agents and are the cause of multiple human diseases. Although the DNA damage responses differ for the different classes of DNA lesions, they share many common general features. One key regulator of the DNA damage

response is the generation of ssDNA. Few DNA damaging agents introduce long ssDNA gaps themselves, but rather does the excision of a bulky adduct or other damages from the DNA template lead to these gaps. Replication stress also leads to an accumulation of ssDNA at the fork, but it remains unclear whether nucleases might participate to increase the gap size beyond a certain threshold that activates the DDR. The same holds true for DNA DSBs that do not contain any ssDNA *per se*. The generation of the structures needed for strand invasion and homology search requires the generation of 3' ssDNA tails by an exonuclease (Zhou and Elledge 2000). The action of the nucleases leads to a DDR by activating the checkpoints. This activation occurs through a pathway initiated with RPA coating of the ssDNA gaps that, in turn, stimulate the binding of ATR-interacting protein (ATRIP). The latter is required for the recruitment of Ataxia-telangiectasia mutated- and Rad3-related (ATR) to sites of DNA damage and for ATR-mediated Chk1 activation in human cells (Zou and Elledge, 2003).

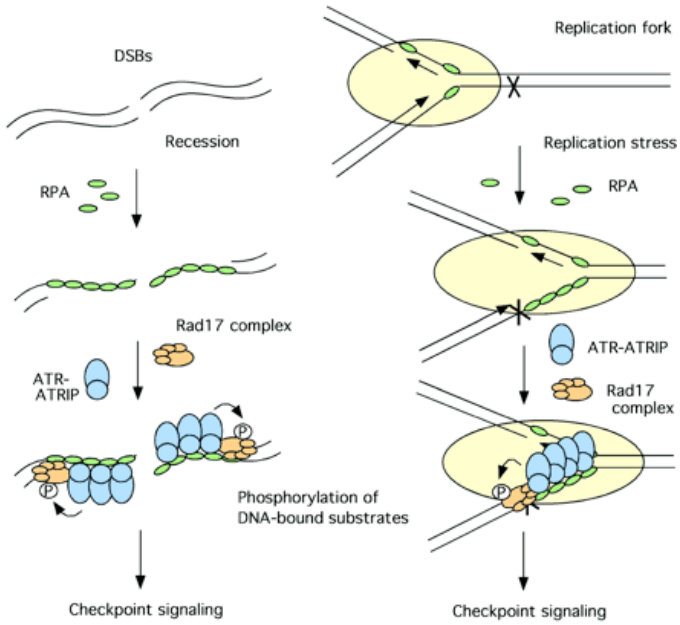


Fig.7 Checkpoint activation in response to DSB's and replication fork stalling

Modified after:

Zou and Elledge

Science. 2003 Jun 6;300(5625):1542-8.

To complete the DDR, ATR additionally phosphorylates substrates such as p53, Brca1 and Rad17 in response to these events. This cascade is highly

conserved, due to its central role in the DDR, which is highlighted by the fact that Ddc2, the yeast ATRIP homologue is recruited to the sites of RPA coated ssDNA in an RPA dependent manner, and the downstream effectors Mec1 (ATR homologue; see table below for complete list of orthologous genes) and Chk1 lead to the same signalling cascade in yeast than in human cells (Zou and Elledge, 2003).

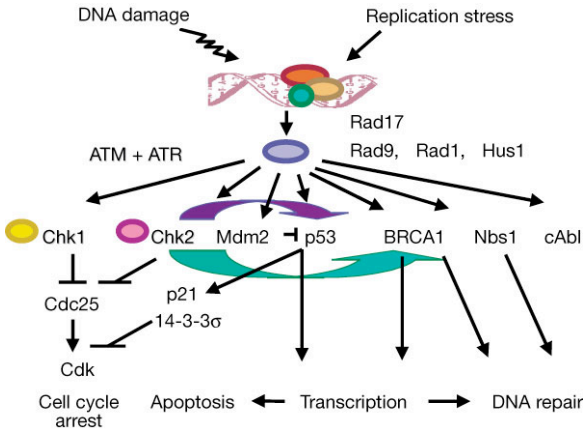


Fig.8 Checkpoint signaling cascade in response to replication stress

Modified after:

Zhou and Ellegdge

Nature. 2000 Nov 23;408(6811):433-9.

1.2.2. Checkpoint kinases

The DNA damage and DNA replication checkpoints are highly conserved cellular surveillance mechanisms permitting cells to maintain genome stability in response to genotoxic stresses. Upon detection of the damage, PIKKs (PI3-kinase like kinases Tel1 and Mec1 in *Saccharomyces cerevisiae* and ATM and ATR in mammals) carry out the initial transduction of the DNA damage signal. Phosphorylation of the downstream targets by the PIKKs facilitates physical protein interactions mediated by the phosphopeptide binding domains, FHA (forkhead-associated) and BRCT (Brca1 C terminus) that are found in numerous DNA damage-response proteins. Among such targets we find Rad9, Mrc1 as well as effector kinases such as Rad53. As a consequence of DNA damage or DNA replication stress, Chk1/Rad53 in *S. cerevisiae* and CHK1/CHK2 in mammals are phosphorylated by PIKKs. These

downstream effector kinases undergo autophosphorylation, where FHA domains mediate self-oligomerization and interactions with mediator proteins such as Rad9 and Mrc1, to become fully active. The formation of these PIKK dependent protein complexes is the juncture at which the DNA damage or replication stress is amplified (Zou and Elledge 2000).

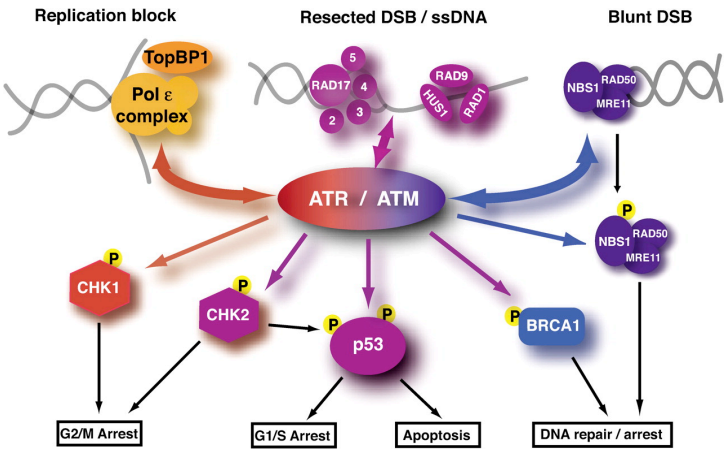


Fig.9 Checkpoint signaling and their downstream targets

Modified after:

Nyberg et al.

Annual Review of Genetics Vol. 36: 617-656

1.2.3. The DNA replication checkpoint

The regulation of DNA replication by checkpoint controls is of key importance for the maintenance of genome stability and, potentially, in cancer therapy. Since fork collapse can lead to chromosome rearrangments, genome instability or cell death, the major role of checkpoint proteins may be to stabilize stalled replication forks. Replication forks slow or stall when they encounter DNA adducts that cause a physical block to the replication fork or when dNTP pools are limiting. Depletion of the nucleotide pool can be achieved by HU treatment, which causes stalling of the replication forks without inducing other damages such as DNA DSBs. Upon disruption of DNA replication, four cellular responses occur: Initiation of replication (origin firing) is blocked, elongation slows down, slowed or stalled replication forks are stabilized and entry into mitosis is blocked. In *S. cerevisiae*, the best-studied model organism,

checkpoint proteins regulate three of these four responses. After DNA damage, the key Mec1 and Rad53 regulators, prevent late origin firing, stabilize stalled replication intermediates and prevent entry into mitosis (Nyberg et al. 2002). Strikingly it remains unclear how slowing of the elongation process is achieved, but it is clear that slowing of elongation does not require the yeast checkpoint. We addressed this question in the study presented here and provide evidence that 14-3-3 proteins are central regulators of the events at stalled replication forks and are especially important for the regulation of the elongation kinetics.

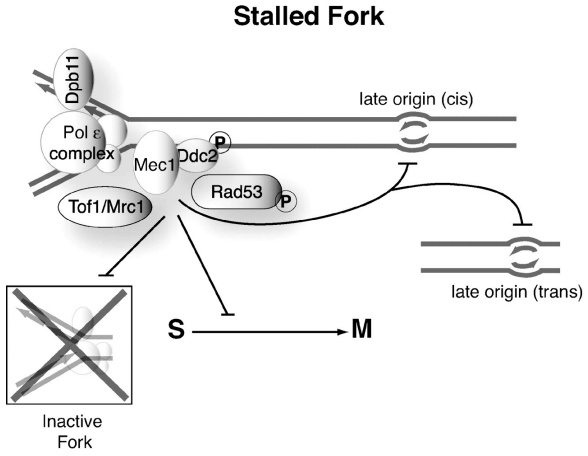


Fig.10 Events occurring at stalled replication forks

Modified after:

Nyberg et al.

Annual Review of Genetics Vol. 36: 617-656

Orthologous DNA damage response proteins			
Protein function	**Mammals**	***S. pombe***	***S. cerevisiae***
Sensors			
RFC1-like	Rad17	Rad17	Rad24
PCNA-like	Rad9	Rad9	Ddc1
	Rad1	Rad1	Rad17
	Hus1	Hus1	Mec3
BRCT-containing	BRCA1	Crb2/Rph9	Rad9
	TopBP1	Cut5	Dpb11
DSB recognition/repair	Mre11	Rad32	Mre11
	Rad50	Rad50	Rad50
	Nbs1		Xrs2
Replication proteins			
recruits polymerases	TopBP1	Cut5	Dpb11
needed for replication		Drc1	Drc1
DNA polymerase	Pol2	Cdc20	Pol2
DNA helicase	BLM, WRN*	Rhq1/Rad12	Sgs1
Topoisomerase	Top3	Top3	Top3
clamp loader	Rfc2–5	Rfc2–5	Rfc2–5
binds ssDNA	Rpa2		Rfa2
Transducers			
PI3-kinases (PIKK)	ATR	Rad3	Mec1
	ATM	Tel1	Tel1
PIKK binding partner	ATRIP	Rad26	Ddc2/Lcd1
Effector Kinases	Chk1	Chk1	Chk1
	Chk2	Cds1	Rad53
Replication fork	—	—	Tof1
stabilizers	Claspin	Mrc1	Mrc1

*WRN—mutated in Werner syndrome.

Table 1 List of DNA damage response proteins

Modified after:

Nyberg et al.

Annual Review of Genetics Vol. 36: 617-656

1.3. DNA Nucleases

1.3.1. General features of DNA nucleases

Nucleases are enzymes capable of cleaving the phosphodiester bonds between nucleotide subunits of nucleic acids. These enzymes play crucial roles in various DNA repair processes, which involve DNA replication, base excision repair, nucleotide excision repair, mismatch repair, and double strand break repair. Nucleases can be further divided into endonucleases and exonucleases. There exist structure specific nucleases such as the flap endonucleases (FENs) and sequence specific nucleases such as the restriction nucleases.

Endonucleases cleave the phosphodiester bond within a polynucleotide chain. Some DNA repair pathways require an incision as first step to grant access of other enzymes to free DNA ends, and this is followed by excision of the damaged structure. One such example is the structure specific endonuclease Mus81/Emi1 that cleaves branched DNA and was recently discovered to be a new fork/junction specific endonuclease.

Restriction enzymes have evolved mainly to protect microorganisms against foreign DNA from invading viruses. These enzymes have the ability to recognise specific DNA sequences and to cut at a predefined position on this sequence. The host DNA remains protected as it undergoes methylation and likewise will not be recognised by the restriction enzyme as a target sequence. The HindII enzyme that originates from Haemophilus influenza and cleaves a specific sequence of six nucleotides always in the middle was the first isolated restriction enzyme and the starting point for the generation of highly useful tools for molecular biology (Tomlinson et al. 2010).

1.3.2. Exonucleases

Exonucleases have the ability to cleave nucleotides one at a time from the end of a polynucleotide chain. It is a hydrolyzing reaction that cleaves the phosphodiester bonds at the 3' or 5' end of DNA or RNA molecules. The diversity of Exonucleases is quite high and most of them are specialized to carry out one specific task. Although some nucleases can only act on the 3' or 5' end

of a DNA or RNA molecule respectively, it is quite frequent the case of complementation of function when an Exonuclease is damaged or absent. These backup Exonucleases usually carry out the reaction with much lower efficiency than the specialized enzyme but their partial redundancy is usually sufficient to prevent any deleterious effects on cell viability or genomic stability. Maintenance of stable replication intermediates when DNA synthesis is impeded requires regulation of several factors, among these, nucleases require particularly fine-tuning (Tran et al. 2004).

1.3.2.1. Exonuclease 1 (Exo1)

Exo1 is a Rad2 family DNA repair nuclease that was originally identified in the fission yeast *Schizosaccharomyces pombe* (Szankasi et al., 1992 and 1995). In vitro, Exo1 was shown to be a structure specific nuclease, which possesses 5'-3' exonuclease and 5' flap endonuclease activity (Lieber, 1997). It is highly conserved throughout the eukaryotic domain and the human *EXO1* gene encodes a protein bearing only 27% identity to its yeast counterpart (Tishkoff et al., 1998). Nevertheless, hEXO1 was shown to complement phenotypes conferred by the deletions of S*accharomyces cerevisiae EXO1* and *RAD27* (Qui et al., 1999) showing that at least certain central aspects of Exo1 function seem to be conserved.

Exo1 catalyzes the removal of mononucleotides from the 5' end of the DNA duplex, showing a strong preference for blunt-ended, 5'-recessed termini and DNA nicks (Lee et al., 1999). The exonucleolytic activity of the enzyme is more efficient on double-stranded DNA (dsDNA) than on single-stranded DNA (ssDNA) (Lee et al., 1999).

Exo1 is implicated in several DNA repair pathways including mismatch repair (MMR), post replication repair, meiotic and mitotic recombination (Fiorentini et al., 1997) (Kirkpatrick et al., 2000) (Tsubouchi and Ogawa, 2000).

The fact that Exo1 is involved in mutation avoidance is due to its role in MMR that has been well characterized. Initially, yeast Exo1 was reported to interact with yeast Msh2 (Tishkoff et al., 1997), an interaction that was also

confirmed for their mammalian counterparts (Schmutte at al., 2001). Mlh1 is another central component of the MMR machinery that was reported in the literature (Schmutte at al., 2001) and confirmed in our yeast two-hybrid screen to interact with Exo1 (see Results, Table 1). Genetic studies demonstrated that Exo1 functions catalytically during MMR (Sokolsky et al., 2000). More recent genetic studies have suggested a catalytic as well as structural role for Exo1 in MMR (Tran et al. 2002). The genetic analyses did not always provide a clear answer to the question if Exo1 is an essential component of MMR. This is probably due to the redundant role of Exo1 to several other nucleases, and maybe even to the redundancy to the exonuclease activity of certain polymerases (Tran et al., 2004). After recognition of a mismatch and the recruitment of all the components of the MMR machinery, Exo1 carries out the excision step that removes the mismatch, thus creating an ssDNA stretch that will serve as a platform for the DNA polymerase. After resynthesis of the excised stretch a ligase will close the remaining nick and likewise complete the MMR (Jiricny 2006).

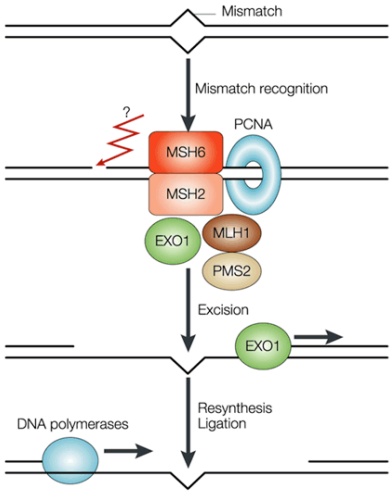


Fig.11 Exo1 plays a structural as well as functional role in MMR

Modified after:

Alberto Martin & Matthew D. Scharff

Nature Reviews Immunology 2, 605-614 (August 2002)

Recent studies have shown that Exo1 is the exonuclease responsible for the processive resection of double strand breaks (DSBs) to generate structures

suitable for homologous recombination (HR) (Zhu et al., 2008). Upon creation of short ssDNA overhangs (end-trimming) by MRE11, a component of the MRN complex (MRX in yeast), Ctip (Sae2 in yeast) promotes DNA end resection (Sartori et al. 2007). The end resection is carried out by Exo1, which generates long 3' single stranded tails from the previously created short ssDNA overhangs. These are the required substrates for binding of the Rad51 recombinase to initiate the homology search and strand invasion steps of recombination (Mimitou and Symington, 2009) and for Rad52-mediated annealing (Mimitou and Symington, 2008).

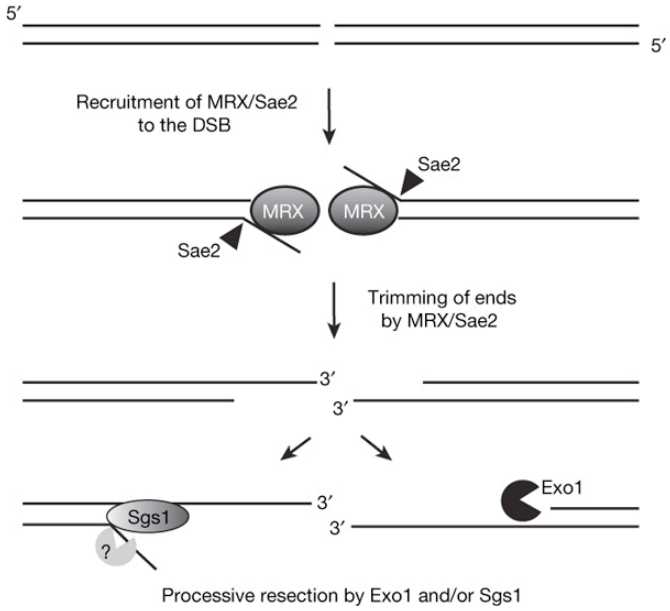


Fig.12 DSB processing by Exo1

Modified after:

Mimitou and Symington

Nature 455, 770-774 (9 October 2008)

The generation of ssDNA is also activating the DNA damage response and likewise leads to a cell cycle arrest in response to DNA damage (Zou and Elledge, 2003). This, in turn, implicates that the regulation of the exonuclease activity of Exo1 is crucial for the control of the checkpoint activation and will reflect on the modulation of the DDR (Morin et al. 2008)

Several lines of evidence suggest that Exo1 plays a crucial role at the replication fork.

Budding yeast Exo1 was previously demonstrated to act redundantly with Rad27 in processing Okazaki fragments during DNA replication (Qui et al., 1999, Tran et al. 2002). More recently, Exo1 was shown to be recruited to stalled replication forks where it counteracts fork reversal through the resection of newly synthesized strands and the resolution of the sister chromatid junctions (Cotta-Ramusino et al., 2005).

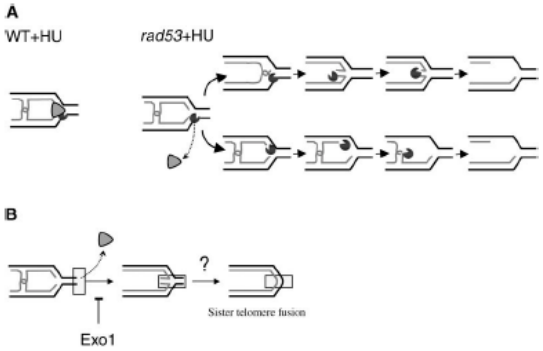


Fig.13 Exo1 processes stalled replication forks and counteracts fork reversal
Modified after:
Cotta-Ramusino
Molecular Cell, Vol. 17, 153–159, January 7, 2005

In mammalian cells, EXO1 activity is controlled by post-translational modifications, with ATR-dependent phosphorylation targeting it to ubiquitin-mediated degradation upon replication fork stalling (El-Shemerly et al., 2005; El-Shemerly et al., 2007), and ATM-dependent phosphorylation apparently restraining its activity to favour RAD51 loading during homologous recombination (Bolderson et al. 2009). Analogously, Mec1-dependent phosphorylation of yeast Exo1 was recently shown to down regulate its activity at uncapped telomeres (Morin et al. 2008). This combined evidence highlights the concept that Exo1 nucleolytic activity is tightly controlled under DNA replication stress and other cellular responses to DNA damage. Substantiating this idea, studies in budding yeast showed that *EXO1* deletion suppresses the sensitivity of *rad53* mutant cells to genotoxic agents that cause reversible or irreversible stalling of the replication forks (Segurado and Diffley 2008), and this suppression was demonstrated to depend on the loss of Exo1 nuclease activity. Interestingly, deletion of *EXO1* is able to suppress the genotoxic sensitivity caused by loss of Rad53 function, but not the one ascribed to

mutations in the upstream kinase Mec1, indicating separable roles in replication fork stabilization for these two kinases (Segurado and Diffley 2008).

1.4. 14-3-3 Proteins

The highly conserved eukaryotic 14-3-3 protein family establishes phosphorylation-dependent interactions and modulates the function of proteins involved in processes such as metabolism, protein trafficking, signal transduction, apoptosis and cell-cycle (Gardino, 2006; Morrison 2009). Although several phosphospecific-binding domains are known, the 14-3-3 proteins usually recognize their target proteins in a specific manner via a discrete phosphoserine or phosphotreonine motif. The two consensus motifs are RSXpS/pTXP and RXXXpS/pTXP, where R is argenine, S is serine, P is proline, X is any amino acid and pS/pT is phosphoserine or phosphothreonine, respectively (Muslin et al., 1996). In addition to the peptide binding groove, other regions of the 14-3-3 surface can determine substrate binding specificity (Wilker et al., 2005). Nevertheless, also "imperfect" sites may be sufficient to bind to the 14-3-3 dimer and phosphoindependent interactions, which do not require the above mentioned consensus sequence, have additionally been reported (Bridges and Moorhead 2005). 14-3-3's are very abundant, small 30 kDa acidic proteins that are expressed in all eukaryotic cells and tissues. Their name is due to their elution and migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis (Aitken 2006). Seven 14-3-3 isoforms exist in mammalian cells - β, γ, ε, σ, ζ, τ, η, but only two in unicellular organisms such as yeast. In *Saccharomyces cerevisiae*, there are two isoforms, the major isoform Bmh1 (Brain Modulosignaling Homologue 1) and the minor isoform Bmh2. Structural analysis showed that 14-3-3 proteins self-assemble into flexible homo- and hetero-dimers that form a central groove able to adapt two extended peptides of varying size (Xiao 1995; Gardino, 2006). This feature confers them the ability to act as adaptor proteins that integrate signals from different pathways (Braselmann 1996; Bridges and Moorhead 2005). 14-3-3 proteins can exert structural effects on their target proteins. As

depicted on panel A below, 14-3-3 proteins can induce conformational changes to their target proteins upon binding. 14-3-3 binding can also result in the occlusion of a specific region on the target and likewise mask for example an active site as it is shown in panel B. And finally, as shown in panel C, binding of 14-3-3 proteins can lead to the colocalization of two proteins, where the 14-3-3 dimer acts as a docking platform (Bridges and Moorhead 2005).

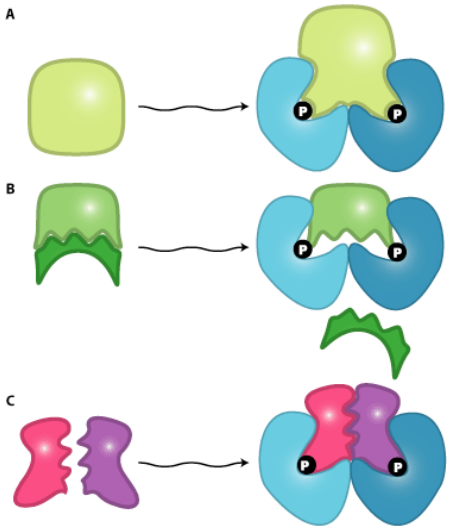


Fig. 14 14-3-3 proteins act as a docking platform
Modified after:
Bridges and Moorhead
Sci. STKE, 9 August 2005

14-3-3 proteins can bind cruciform DNA, a structure that was shown to form at yeast origins of replication (Yahyaoui et al., 2007), and are able to associate with replication initiation proteins such as Mcm2 and Orc2 (Yahyaoui 2009).

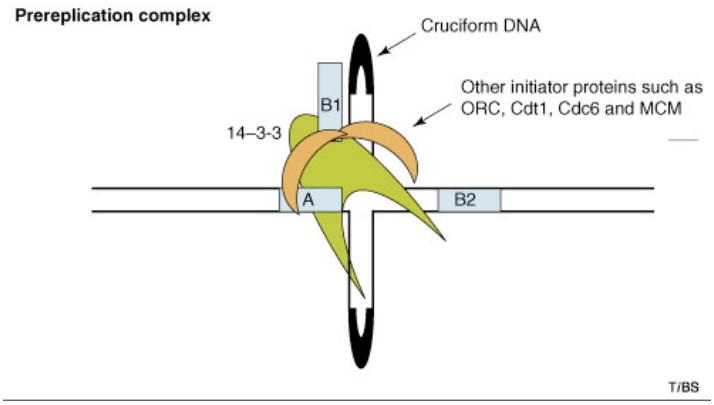


Fig.15 14-3-3 Proteins can bind cruciform DNA

Modified after:

Hadjopoulos

TiBS, Volume 33, Issue 1, January 2008, Pages 44-50

Upon DNA damage and DNA replication stress, 14-3-3 proteins are required for cell cycle restart, suppression of genomic instability and viability (Lottersberger et al., 2003). Moreover, 14-3-3 proteins genetically and physically interact with the checkpoint protein Rad53 and genetically with the checkpoint kinase Dun1. The physical interaction with Rad53 only occurs in the presence of the DNA damaging agent MMS (Methyl methanesulfonate). 14-3-3 proteins directly facilitate Rad53 function *in vivo* by stabilizing an active form of the kinase (Usui and Petrini, 2007). These interactions may be an explanation for the previously observed checkpoint defects in 14-3-3 mutant yeast cells (Lottersberger et al. 2003). The yeast 14-3-3 proteins were also shown to interact with the acetyltransferases and deacetylases Esa1 and Rpd3 upon replication perturbations (Lottersberger et al., 2007). Taken together, these data point to an important role of 14-3-3 during replication stress, though their exact mechanism of action remains unknown.

2. RESULTS

2.1. 14-3-3 Proteins Regulate Exonuclease 1-Dependent Processing Of Stalled Replication Forks

Kim Engels, Michele Giannattasio, Marco Muzi-Falconi, Massimo Lopes and Stefano Ferrari

(manuscript submitted to PLOS genetics)

This manuscript describes a novel function of 14-3-3 proteins during replication stress. Our work identifies 14-3-3 proteins as novel interaction partner of Exonuclease1, an enzyme with an increasingly important role in DNA metabolism, recently claimed to participate in the onset of genome instability. 14-3-3 proteins were also previously shown to play a role in protecting genome stability, although the underlying mechanism had escaped identification. This manuscript describes an unprecedented link in the field, showing that an evolutionarily conserved pathway through which 14-3-3 proteins maintain genome integrity after replication stress relies on the control of Exo1 activity at stalled replication forks. Additionally this manuscript provides evidence for the control of replication fork progression under limiting nucleotide concentrations by 14-3-3 proteins, and poses them as central regulators of events at stalled replication forks.

I contributed to this study by designing and performing the research and by writing the manuscript.

14-3-3 Proteins Regulate Exonuclease 1-Dependent Processing Of Stalled Replication Forks

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Running Title: 14-3-3 regulate Exo1 at replication forks

Keywords: 14-3-3, Exonuclease 1, Replication fork, Rad53

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ABSTRACT

Replication fork integrity, which is essential for the maintenance of genome stability, is monitored by checkpoint-mediated phosphorylation events. 14-3-3 proteins are able to bind phosphorylated proteins and were shown to play an undefined role under DNA replication stress. Exonuclease 1 (Exo1) processes stalled replication forks in checkpoint-defective yeast cells. We now identify 14-3-3 proteins as *in vivo* interaction partners of Exo1, both in yeast and mammalian cells. Yeast 14-3-3-deficient cells fail to induce Mec1-dependent Exo1 hyperphosphorylation and accumulate Exo1-dependent ssDNA gaps at stalled forks, as revealed by electron microscopy. This leads to persistent checkpoint activation and exacerbated recovery defects. Moreover, using DNA bi-dimensional electrophoresis we show that 14-3-3 promote fork progression under limiting nucleotide concentrations. We propose that 14-3-3 proteins assist checkpoint-mediated phosphorylation of Exo1 and additional unknown targets, promoting fork progression, stability and restart in response to DNA replication stress.

INTRODUCTION

DNA lesions can cause stalling and collapse of the replication fork and lead to chromosome breaks, mutations, genome rearrangements and eventually cancer (Branzei and Foiani 2010). To prevent this, a replication checkpoint has evolved as surveillance mechanism to control components of the replisome (Muzi-Falconi et al. 2003) and to allow coordinating replication with cell cycle progression and DNA repair. Maintenance of stable replication intermediates when DNA synthesis is impeded requires regulation of several factors. Among these, nucleases require particularly fine-tuning.

Exo1 is a Rad2 family DNA repair nuclease able to remove mononucleotides from the 5' end of the DNA duplex (Lee and Wilson 1999) that was originally identified in the *S. pombe* (Szankasi and Smith 1992) and subsequently in humans (Tishkoff et al. 1998). Exo1 is implicated in several DNA repair pathways including mismatch repair, post replication repair, meiotic and mitotic recombination and double strand breaks repair (Szankasi and Smith 1995; Fiorentini et al. 1997; Kirkpatrick et al. 2000; Tsubouchi and Ogawa 2000; Mimitou and Symington 2009). *S. cerevisiae* Exo1 acts redundantly with Rad27 in processing Okazaki fragments during DNA replication (Qiu et al. 1999). More recently, Exo1 was shown to be recruited to stalled replication forks where it counteracts fork reversal (Cotta-Ramusino et al. 2005). Human EXO1 activity is controlled by post-translational modifications, with ATR-dependent phosphorylation targeting it to ubiquitin-mediated degradation upon replication fork stalling (El-Shemerly et al. 2005; El-Shemerly et al. 2008), and ATM-dependent phosphorylation apparently restraining its activity during homologous recombination (Bolderson et al. 2010). Analogously, Mec1-dependent phosphorylation inhibits yeast Exo1 activity at uncapped telomeres (Morin et al. 2008). Studies in budding yeast showed that *EXO1* deletion suppresses the sensitivity of *rad53*, but not *mec1*, mutant cells to agents causing reversible or irreversible stalling of replication forks (Segurado and Diffley 2008). Taken together, this evidence indicates that Exo1 activity is tightly controlled under DNA replication stress and DNA damage.

Eukaryotic 14-3-3 are highly conserved proteins that establish phosphorylation-dependent interactions and modulate the functions of proteins involved in processes such as metabolism, protein trafficking, signal transduction, apoptosis and cell-cycle (Morrison 2009). Seven 14-3-3 isoforms exist in mammalian cells, but only two in yeast. Structural analysis showed that 14-3-3 proteins self-assemble into flexible homo- and hetero-dimers forming a central groove that is able to adapt two extended peptides (Xiao et al. 1995; Gardino et al. 2006). This feature confers them the ability to act as adaptors that integrate signals from different pathways (Braselmann and McCormick 1995; Bridges and Moorhead 2005). 14-3-3 proteins can also bind cruciform DNA (Yahyaoui et al. 2007) and replication initiation proteins such as Mcm2 and Orc2 (Yahyaoui and Zannis-Hadjopoulos 2009). Upon DNA damage and DNA replication stress, 14-3-3 proteins are required for cell cycle restart, suppression of genomic instability and viability (Lottersberger et al. 2003). Moreover, 14-3-3 proteins genetically and physically interact with the checkpoint protein Rad53 (Usui and Petrini 2007) as well as the acetyltransferases and deacetylases Esa1 and Rpd3 upon replication perturbations (Lottersberger et al. 2007). Although these data point to an important role of 14-3-3 during replication stress, the exact mechanism of 14-3-3 action remains unknown.

In this study, we identify 14-3-3 as novel interaction partners of Exo1 and demonstrate that they regulate phosphorylation of the nuclease. We provide evidence for an accumulation of Exo1-dependent ssDNA gaps at stalled forks in yeast 14-3-3 deficient cells and we show that this causes persistent checkpoint activation and recovery defects. We also show that 14-3-3 proteins control progression and stability of replication forks under conditions of limiting nucleotide availability. Taken together, our data demonstrate that 14-3-3 have a crucial role in regulating the function of proteins at stalled forks, among which Exo1 is a key target.

RESULTS AND DISCUSSION

14-3-3 proteins interact with EXO1

To identify novel interaction partners for human EXO1 we designed a yeast two-hybrid screen with GAL4-bait fusion proteins that contain either full-length EXO1 or DN-EXO1 ($EXO1_{366-846}$), which lacks the entire catalytic domain. Since the former was not expressed (data not shown), we used the latter to screen a blood peripheral cDNA library. Three 14-3-3 proteins were the highest hits (Supplemental Table 1), with the b- being more represented than the e- and z-isoform. The presence of an established EXO1 binding protein among the hits, MLH1 (Supplemental Table 1), confirmed the reliability of this screen.

To independently verify these data, we performed co-immunoprecipitation experiments. Given the low abundance of EXO1 in the cell (El-Shemerly et al. 2005), we transiently transfected HEK-293 cells with an Omni-tagged EXO1 construct (El-Shemerly et al. 2005) and immunoprecipitated the expressed protein using a pan-14-3-3 antibody. The data showed that Omni-EXO1 and 14-3-3 proteins could be recovered as a complex (Fig. 1A).

To assess the physiological significance of the EXO1/14-3-3 interaction we selected *S. cerevisiae*, a system where only two 14-3-3 proteins are present, namely Bmh1 and Bmh2. In preliminary experiments we examined whether yeast Exo1 and 14-3-3 proteins interact. A C-terminal Myc- or a HA-tag was added to the endogenous *EXO1* or *BMH1*/*BMH2* genes, respectively. Immunoprecipitation experiments showed that Exo1 formed complexes with Bmh1 or Bmh2 in a HU-dependent manner (Fig. 1B).

Taken together, these data suggest that the EXO1/14-3-3 interaction is conserved from yeast to mammalian cells. While the interaction is HU-independent in mammalian cells, it requires HU in yeast. This may reflect the different modes of EXO1 regulation in the two systems (El-Shemerly et al. 2008; Morin et al. 2008).

14-3-3 deficient cells cannot restart stalled replication forks, but their recovery defect is partially suppressed by *EXO1* deletion

Genetic and flow cytometric analysis evidenced the sensitivity of 14-3-3-deficient cells to DNA replication stress, with distinct *bmh1* (*bmh2D*) alleles showing different defects upon nucleotide depletion (HU) or treatment with DNA damaging agents (UV or methylmethansulfonate, MMS) (Lottersberger et al. 2003). However, despite the evidence that 14-3-3 proteins bind origins of replication and cruciform DNA (Alvarez et al. 2002), suggesting a regulatory role in DNA replication (Yahyaoui and Zannis-Hadjopoulos 2009), the issue of possible direct involvement of 14-3-3 in fork stability or processing under genotoxic stress conditions remained to be clarified. Given the comprehensive molecular characterization of yeast Exo1 as component of the replisome and of its role, in checkpoint defective cells, in the processing of forks stalled by nucleotide depletion (Cotta-Ramusino et al. 2005), we focused on the *bmh1-280 bmh2D* allele (*bmh1bmh2* hereafter), which shows normal cell cycle progression in unperturbed conditions, but selective sensitivity and cell cycle recovery defects in response to HU (Lottersberger et al. 2003). We thus asked whether these defects reflect a direct role of 14-3-3 proteins at replication forks and whether Exo1 is also implicated in these processes. We performed neutral-neutral bidimensional gel electrophoresis (2D gel) on the early origin of replication ARS305 - known to be activated in HU-treated cells (Lopes et al. 2001) - and observed that replication forks were still present close to the origin in *bmh1bmh2* cells 60 min after HU removal (Fig. 2A). Thus, although the 2D gel pattern looked normal in these cells, their forks failed to resume DNA synthesis, suggesting that misregulation of the replisome, without dramatic physical processing of the forks, might be sufficient to prevent fork restart. This effect was not detectably suppressed by *EXO1* deletion (Fig. 2A).

Flow cytometric analysis of HU-released cells confirmed the slow recovery of the *bmh1bmh2* strain and showed that lack of Exo1 *per se* did not alter the pattern of cell cycle progression (Fig. 2B). On the other hand, *EXO1* deletion in a *bmh1bmh2* background led to a partial rescue of the recovery defect, particularly evident 120 min after release from HU (Fig. 2B). This evidence prompted us to ask whether *EXO1* deletion in this background may affect Rad53 activity. Western blot analysis showed that, compared to wild type cells, Rad53 was hyperphosphorylated in HU-treated *bmh1bmh2* cells and that its

dephosphorylation was retarded during the HU-recovery phase (Fig. 2C), thus correlating with the described replication restart defect. Importantly, deletion of *EXO1* in 14-3-3-deficient cells re-established to a great extent the pattern of rapid Rad53 dephosphorylation in the recovery phase (Fig. 2C), substantiating the flow cytometry data (Fig. 2B). Overall these data suggest that 14-3-3 proteins are directly implicated in the effective restart of stalled DNA replication forks. In their absence, Exo1 activity does not directly impact the rate of fork restart, but contributes to induce slow checkpoint inactivation and impaired cell cycle resumption.

Reversible Exo1 phosphorylation in response to HU is dependent on 14-3-3 proteins

Exo1 is controlled in a phosphorylation-dependent manner upon replication fork stalling in mammalian cells (El-Shemerly et al. 2005) and upon a variety of genotoxic insults in yeast (Morin et al. 2008). We obtained evidence that yeast Exo1 is phosphorylated in a Mec1-dependent manner also in response to HU (Fig. 3A). Notably, the improved resolution of Exo1 phospho-forms over previously published work allowed us visualizing the complete pattern of Exo1 phosphorylation in response to replicative stress (Fig. 3A and 3B).

Next, we asked whether 14-3-3 proteins might be involved in the regulation of Exo1 phosphorylation. Western blot analysis showed that in 14-3-3-deficient cells Exo1 was not phosphorylated to the same stoichiometry observed in wild type cells (Fig. 3B, 90 min). Moreover, the rate of Exo1 dephosphorylation upon recovery from HU was considerably reduced in mutant cells, with Exo1 being completely dephosphorylated in wild type but not in 14-3-3-deficient cells (Fig. 3B, 120 min). Defective Exo1 phosphorylation in HU-treated 14-3-3-deficient cells is not an indirect consequence of defective checkpoint activation, as under these conditions Rad53, another Mec1-dependent checkpoint target, is promptly phosphorylated (Fig. 2C). Since phosphorylation restrains yeast Exo1 activity (Morin et al. 2008), we propose that 14-3-3 proteins play an important role in the dynamic control of Exo1 activity upon DNA replication stress and may act as platform for Exo1 phosphorylation to take place.

Exo1 is responsible for the accumulation of ssDNA gaps behind the fork in *bmh1bmh2* cells

As Exo1 activity and Rad53 phosphorylation have been linked to the processing of stalled DNA replication forks, we decided to assess whether defective Rad53 and Exo1 phosphorylation in 14-3-3-deficient cells could reflect changes in the fine architecture of stalled forks. To answer this question, we synchronized the cells in G1, released them for 1h in YPD medium containing 0.2 M HU and examined replication intermediates by electron microscopy (EM) under non-denaturing conditions (Lopes 2009). For each strain, about 100 replication intermediates were analyzed in duplicate. 14-3-3-deficient cells showed a dramatic accumulation of ssDNA gaps behind the replication fork (Fig. 4A). Statistical analysis indicated that approximately 50% of all replication intermediates analyzed contained one or more ssDNA gaps (Fig. 4B). Interestingly, deletion of *EXO1* in the *bmh1bmh2* background completely suppressed this phenotype, leading to a reduction of the ssDNA gaps behind the fork to a level similar to wild type or *exo1Δ* cells (Fig. 4B). The comparison of ssDNA gaps length scored by EM evidenced a striking difference: whereas *bmh1bmh2* cells displayed a significant number of large size gaps (>0.5 Kb), the latter were absent in *bmh1bmh2 exo1Δ* cells (Fig. 4C). These data suggest that 14-3-3 proteins are required to prevent unscheduled Exo1 activity behind stalled replication forks in a checkpoint-proficient background. The implications of these observations are of great significance. Since Exo1 is a 5'-3' exonuclease, a loose control of its activity may render DNA synthesis more discontinuous in conditions of replicative stress. Although additional work is required to directly address this point, it is conceivable that continuous polymerase stall due to insufficient deoxynucleotide levels might lead to increased repriming events, thus raising the number of 5'-ends available for processing by Exo1. In this setting, a strict control of Exo1 activity would be needed to limit damage. The resolution limit of 50-70 nucleotides may have impaired detection of nicks/small gaps in this as well as in previous EM studies with HU (Sogo et al. 2002). Such structures, however, become visible in 14-3-3-deficient cells, where the unleashed Exo1 activity would enlarge gaps above the detection limit.

Defective fork progression in 14-3-3 defective cells is independent on *EXO1*

Replication recovery defects have been previously described and usually reflect replication fork collapse detectable by 2D gel analysis (Lopes et al. 2001). On the contrary, stalled replication forks in 14-3-3 deficient cells, albeit unable to restart DNA synthesis and abnormally processed by Exo1 activity, upon prolonged HU treatment show a 2D gel pattern indistinguishable from that of wild type cells. We thus decided to investigate in more detail the structure and progression of these forks, performing 2D gel analysis at different time points after HU addition. To this end, cells synchronized in G1 by a-factor were released into medium containing HU and replication intermediates were examined by 2D gels. Fig. 5B shows the probes designed to visualize replication fork progression in a region of Chromosome III that contains, besides the early active origin ARS305 (Newlon and Theis 1993), a contiguous passively replicated region (Part A) and a region including the dormant origin ARS301 (Part D) (Lopes et al. 2001). As compared to wild type, *bmh1bmh2* cells showed the same kinetics of origin firing, albeit with slightly lower efficiency as revealed by the intensity of the bubble arc at 30 min (Fig. 5C). Progression of the forks in HU from ARS305 across the region of Part A (~5 Kb to the left of ARS305) was completed after 2-3h in wild type cells, with the peak of intermediates detectable after ~1h. In *bmh1bmh2* cells the first intermediates appeared on this region with 30 min delay, whereas the peak of intermediates was delayed of ~2h as compared to wild type cells (Fig. 5C), indicative of a significant decrease in the rate of the replication fork progression in HU.

It was previously shown that yeast 14-3-3 proteins bind to the checkpoint kinase Rad53 and directly influence its DNA damage-dependent functions (Usui and Petrini 2007). Therefore, we asked whether the slow fork progression in *bmh1bmh2* cells might be solely due to checkpoint defects. To address this issue, we used checkpoint defective Rad53-mutant cells (*rad53-K227A*). The latter displayed striking differences when compared to *bmh1bmh2* cells. Both the destabilization of replication intermediates (ARS305 and Part A) and the uncontrolled firing of dormant origins displayed by *rad53-K227A* cells (Part D)

(Lopes et al. 2001), were absent in *bmh1bmh2* cells (Fig. 5C). Furthermore, electron microscopy did not display any fork reversal or accumulation of ssDNA at replication forks, typical of HU-treated *rad53* cells (Sogo et al. 2002) (data not shown). Finally, drop assays (Supplemental Fig. S1) and 2D gel analysis (Supplemental Fig. S2C, F and H) revealed synergistic effects of 14-3-3 and Rad53 on both survival and fork stability. Overall, these data indicate that the phenotype observed in 14-3-3 deficient cells reflects a genuine role of 14-3-3 proteins at replication forks and that 14-3-3 and Rad53 have crucial but distinct roles at HU-challenged forks.

Deletion of *EXO1* partially rescued the HU-sensitivity of *rad53-K227A* cells, but not of a *bmh1 bmh2* strain (Supplemental Fig. S1). Furthermore, in contrast to checkpoint defective cells, where stability of replication intermediates could be rescued by *EXO1* deletion (Cotta-Ramusino et al. 2005), fork progression defects of *bmh1bmh2* cells were not rescued by loss of *EXO1* (Supplemental Fig. S2G). Thus, while the processing defect that leads to accumulation of ssDNA gaps in 14-3-3-deficient cells was completely suppressed by EXO1 deletion, this did not reflect in suppression of HU sensitivity nor of defective fork progression in HU-treated 14-3-3 deficient cells. Altogether this evidence suggests that 14-3-3 proteins might regulate additional targets during replication stress, possibly through modulation of their phosphorylation. This is not unexpected, given the role of 14-3-3 as integrators of signalling pathways (Morrison 2009) and considering the multiplicity of 14-3-3 targets (Jin et al. 2004; Pozuelo Rubio et al. 2004). Our data implicate 14-3-3 proteins as possible central regulator of the checkpoint response. In analogy with previously reported cases (Braselmann and McCormick 1995) and according to structural data on the dynamic nature of 14-3-3 dimers (Yang et al. 2006), one may envisage a role for 14-3-3 proteins as docking clamp tethering Exo1 -and other unknown targets - with the kinase controlling its/their activity. Notably, 14-3-3 proteins were reported to bind Rad53 (Usui and Petrini 2007), one of the candidate checkpoint kinases required for Exo1 phosphorylation (Morin et al. 2008).

In conclusion, this work sheds further light on processes occurring at stalled replication forks, proposing 14-3-3 proteins as central integrators of signals that

regulate fork stability and processing. Challenges lying ahead consist in the identification of components of the replisome, or proteins controlling them, that may be 14-3-3 targets, as well as in the elucidation of the exact mechanism by which 14-3-3 modulate Exo1 activity.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains and cell culture

The yeast strains used in this study are isogenic to W303 and are listed in Supplemental Material. HEK-293 cells were maintained and transiently transfected as described (El-Shemerly et al. 2005).

Yeast two-hybrid screen

The yeast two-hybrid screening was performed with DN-EXO1 ($EXO1_{366-846}$) as bait on a cDNA library generated from human peripheral blood mRNA (a kind gift of I. Stagljar, Toronto, Canada) as described previously (Jiao et al. 2004) and using THY AP4 as reporter strain.

Western Blotting, Immunoprecipitation

To visualize Exo1, an optimized Phos-tag system (5 mM Phos-tag reagent) was employed according to (Kinoshita et al. 2008). Proteins were transferred to nitrocellulose (porablot NCP, 0.45 μm pore size, Machery-Nagel) overnight at room temperature applying constant amperage (200 mA). Additional information about protein extraction and immunoprecipitation can be found in Supplemental Material.

2D Gel Electrophoresis and Electron Microscopy

DNA extraction with the CTAB method and neutral-neutral two-dimensional gel electrophoresis were performed as described (Lopes et al. 2003). Replication intermediates quantification (Lopes et al. 2001) and EM analysis (Lopes 2009) were performed as described.

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FIGURE LEGENDS

Figure 1 - EXO1 interacts with 14-3-3 proteins

(A) HEK-293 cells were transiently transfected with empty vector (-) or pcDNA3.1-Omni-EXO1. Whole cell extracts (WCE, 2.5 mg) were immunoprecipitated with a pan-14-3-3 antibody and proteins were detected as indicated. Input = 50 mg WCE (B) Control yeast culture (wt) or cultures expressing Bmh1-HA Exo1-Myc (1) or Bmh2-HA Exo1-Myc (2) were treated for 3 h with 150 mM HU. WCE (10mg) were immunoprecipitated with the monoclonal antibody to HA and proteins were detected as indicated. CNTL = immunoprecipitation performed in the absence of the antibody. Input = 100 mg WCE.

Figure 2 - Pattern of HU recovery in wild type, *exo1*, *bmh1-280 bmh2Δ* and *bmh1-280 bmh2Δ exo1Δ* strains

(A) Representative 2D gels of RI at ARS305 analyzed 1h upon release from HU. (B) Time-course flow cytometric analysis of the DNA content in the indicated strains upon recovery from a HU-arrest. (C) Western blot analysis of Rad53 phosphorylation in HU-arrested cells and during the recovery phase.

Figure 3 - Phosphorylation pattern of Exo1 in response to HU in wild type, *mec1D*, and *bmh1-280 bmh2Δ* strains

(A) Western blot analysis of Exo1 phosphorylation in HU-arrested cells of the indicated strains. (B) Western blot analysis of Exo1 phosphorylation in HU-arrested cells and during the recovery phase of the indicated strains.

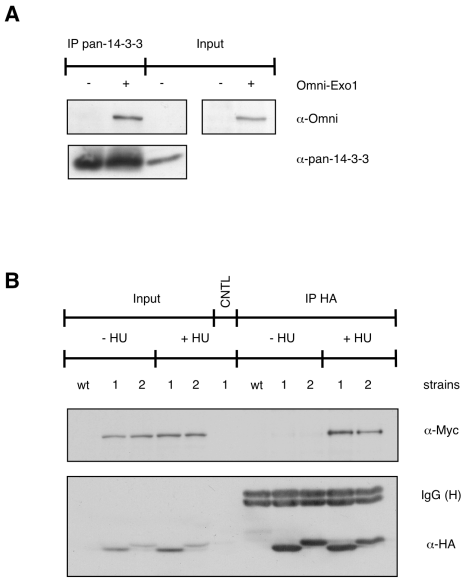
Figure 4 - Exo1-dependent generation of ssDNA gaps in *bmh1 bmh2* mutant cells

(A) Representative replication intermediates visualized by electron microscopy in *bmh1bmh2* cells released synchronously from G1 phase in HU 0.2 M for 1h: the magnified inset (asterisk) shows a representative ssDNA gap located behind the replication fork. Black arrows: ssDNA gap at the fork; White arrows:

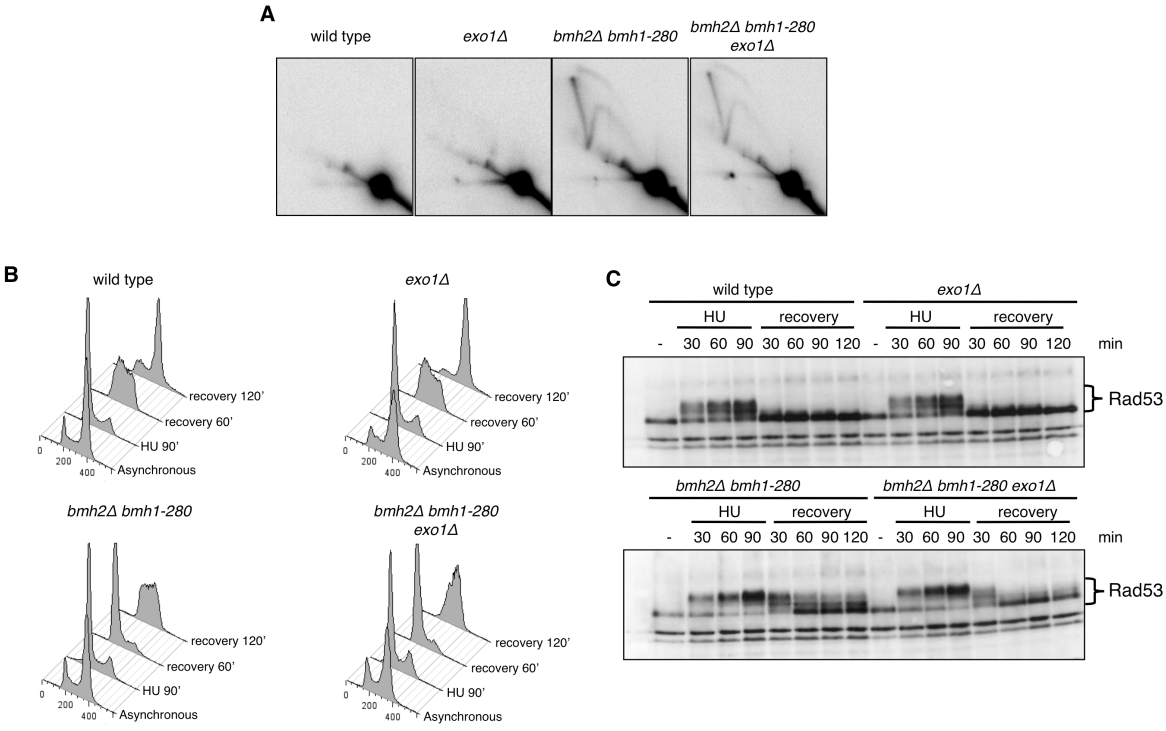
internal ssDNA gap located behind the fork. (B) (C) Statistical analysis of ssDNA gaps number and length.

Figure 5 - 2D gel analysis of replication intermediates from wild type, *bmh1-280 bmh2Δ* and *rad53-K227A* strains

(A) Schematic representation of replication intermediates visualized by 2D gel electrophoresis. (B) Chromosome III region adjacent to ARS305 with indication of the probes used in 2D gel analysis. (C) Time-course resolution of replication intermediates obtained from the indicated strains grown in the presence of 0.2 M HU.



Engels et al. Fig. 1



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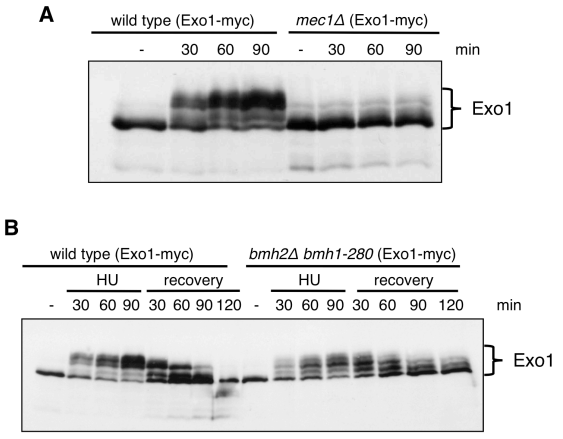
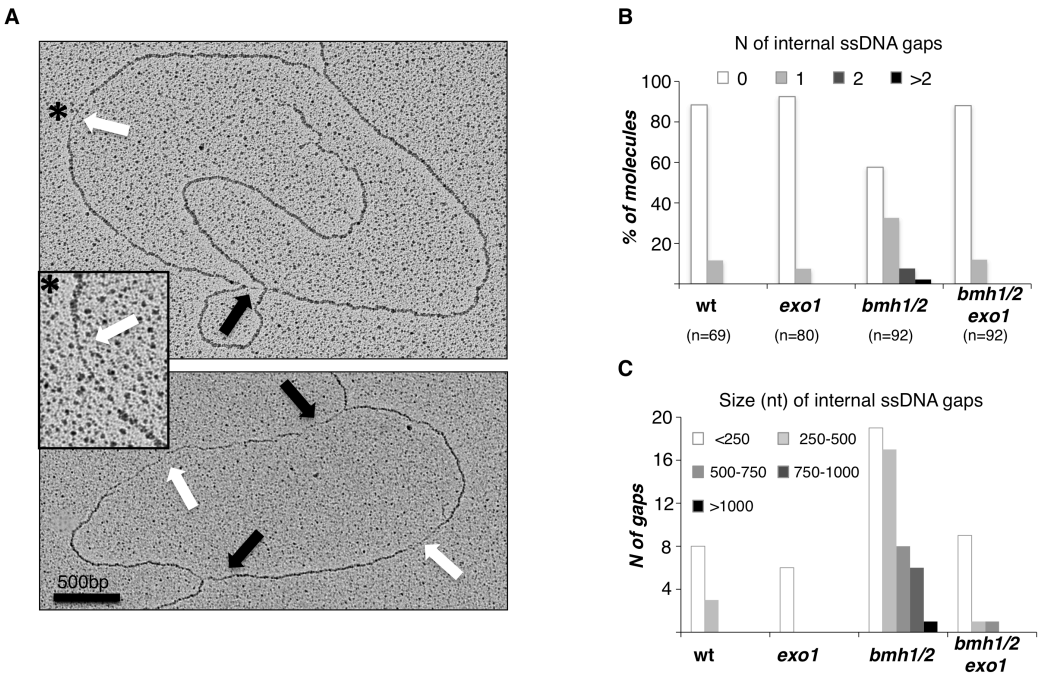
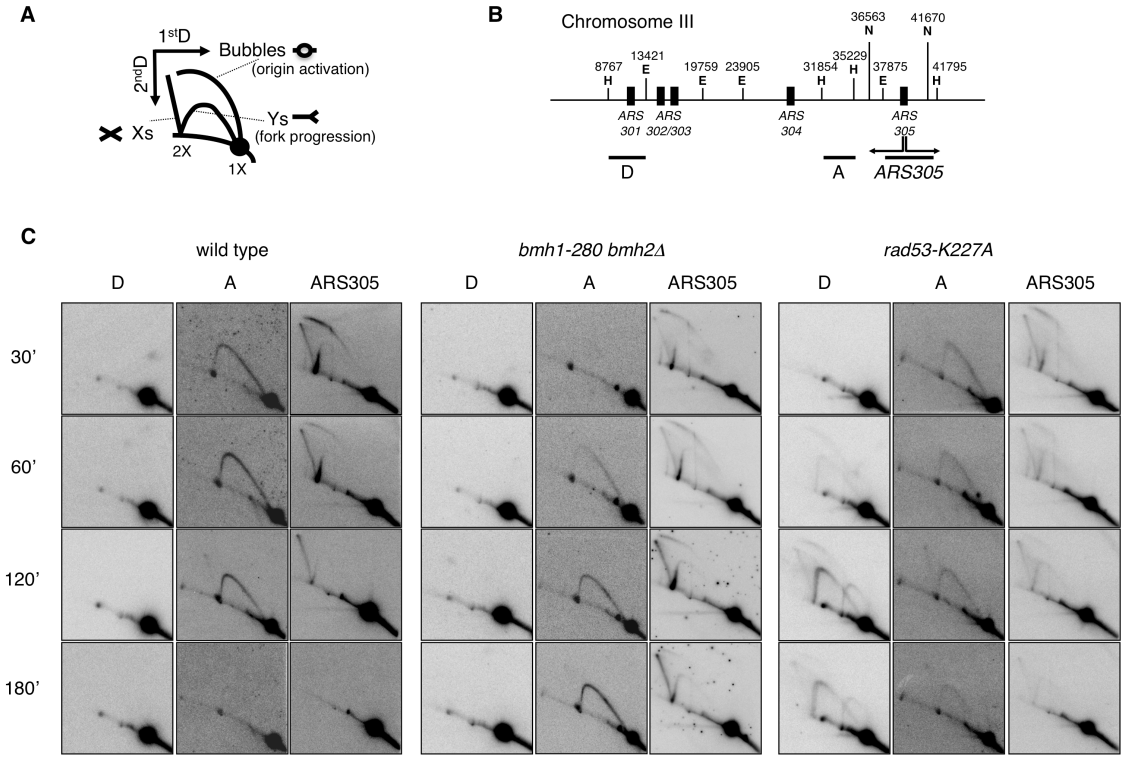


Engels et al. Fig. 3



Engels et al. Fig. 4



Engels et al. Fig. 5

2.2. Yeast two-hybrid screen identifies several novel human Exo1 interaction partners

To identify novel interaction partners for human EXO1 we performed a yeast two-hybrid screen. To this end, we constructed LexA-bait fusion proteins containing either full-length EXO1 or DN-EXO1 ($EXO1_{366-846}$), which lacks the entire catalytic domain. By sequencing we verified that the cloning resulted in correct in frame junction of the bait sequences to the LexA DNA binding domain. Since the full length EXO1 was not expressed from the bait plasmid we used the properly expressed DN-EXO1 construct to perform the Yeast two-hybrid experiments (Fig.16A). We tested the expressed bait construct (pLexA-DN-EXO1) for self-activation by co-transforming it into the THY.AP4 yeast reporter strain with the empty prey plasmid (pACT2). To assess the level of self-activation of our bait construct we grew the transformants on different selective plates (SD-TL, SD-TLH and SD-TLAH). The DN-EXO1 bait construct did not show any self-activation as demonstrated by the absence of colonies on SD-TLH and SD-TLAH plates (Fig.16B).

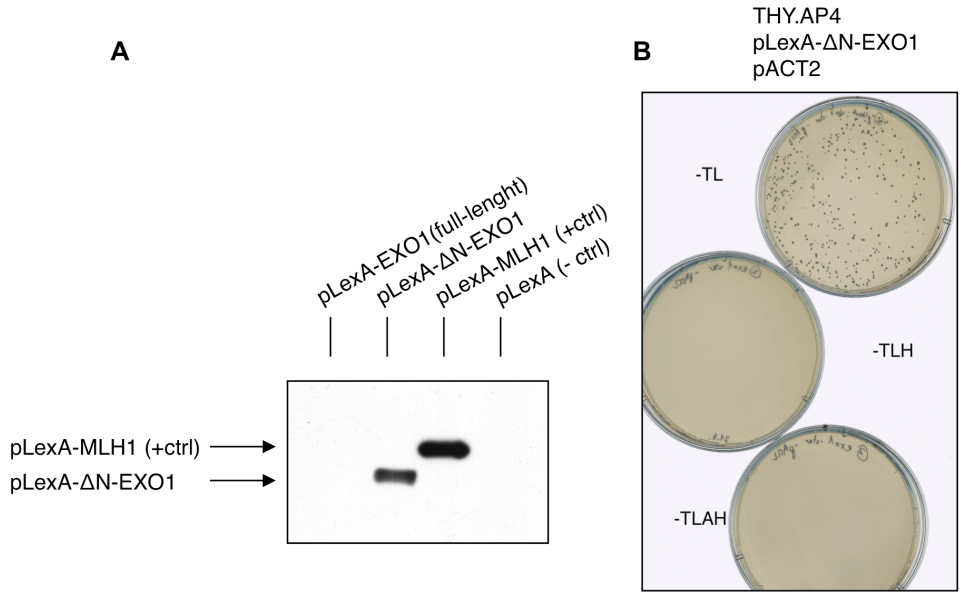


Fig.16 Validation of the tools for the yeast two-hybrid screen. A) Western Blot analysis to test the expression of the bait construct in the reporter strain THY.AP4. The pLexA-ΔN-EXO1 is well expressed and will subsequently be used for the screening approach B) Self-activation assay. Growth on selective plates -TLH and –TLAH confirms that the bait construct pLexA-ΔN-EXO1 is not self activating (in the absence of an interaction) and likewise can be used to screen a cDNA library

In order to increase the screening stringency we ran a pilot screen to determine the amount of 3-amino-1,2,4-triazol (3-AT) that may have to be included to the selection plates. 3-AT modulates the sensitivity of the HIS3 reporter gene by acting as a competitive inhibitor of the His3 protein. Since we could hardly spot any background colonies growing on the selective plates with increasing 3-AT concentrations or without, we concluded that we do not need to complement the selection plates to screen our DN-EXO1 bait construct (data not shown). For the Yeast two-hybrid screen 56μg of a blood peripheral cDNA prey library were transformed into the THY.AP4 reporter strain expressing our DN-EXO1 bait construct. The transformed cells were plated on 32 150mm diameter SD-TL and SD-TLAH dishes each. From the control plates we calculated the total number of transformants ($1{,}1*10^6$) and likewise we achieved a transformation efficiency of $3{,}93*10^4$ (clones/μg DNA). From the SD-TLAH

selection plates we isolated 135 positive clones and tested them for ß-Galactosidase activity. Only the clones that were positive for both parameters, the growth and the colorimetric markers, were further considered. Their plasmids were isolated by standard procedures from the yeast cells and re-transformed into Escherichia coli (E. coli), where we could specifically select and amplify the prey plasmids. These isolated prey plasmids were sequenced and the obtained sequences were subjected to a blast search to uncover the putative interacting proteins. Like any genetic selection system, the Yeast two-hybrid screening system will isolate a number of false positives. These clones will result in a $his^+/ade^+/lacZ^+$ phenotype independent of a true interaction. The sequences obtained from the blast search allowed us to exclude all the clones that were out of frame or inverted. Additionally we could discard the clones containing typical false-positives like small "sticky" peptides. The remaining twelve potentially interesting positive clones were subjected to a bait dependency assay. The bait dependency test is normally performed to confirm the interactions found in the screening approach and serves to eliminate those preys that non-specifically interact with any co-expressed bait, the so-called false positives. For this approach the plasmids containing the putative novel interacting protein were retransformed into the reporter strain expressing either the DN-EXO1 bait construct or, as a negative control, a non-cognate bait construct: LaminC. The resulting co-transformants were assayed again for activation of reporter genes and in this manner false-positive interaction partners could be removed (Fig17).

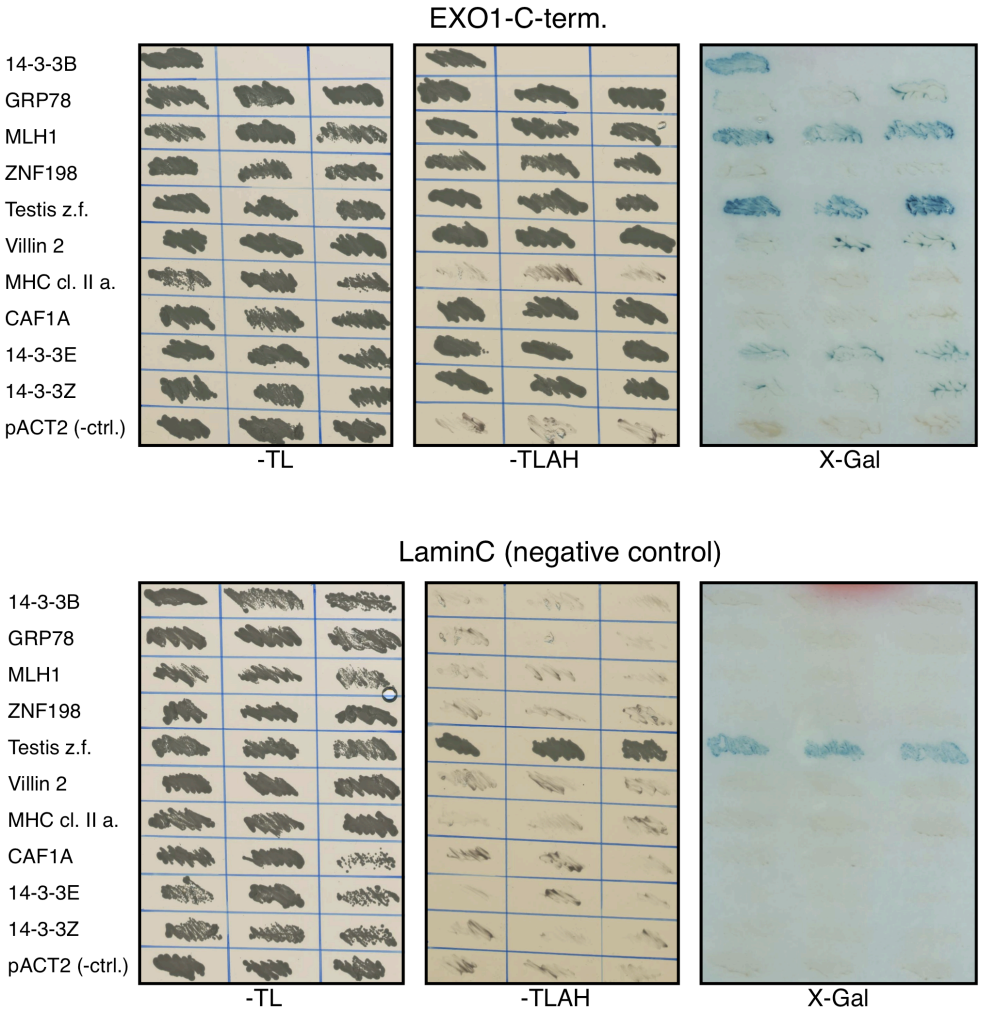


Fig.17 Bait dependency assay confirming the specificity of the interaction partners found in the Y2H screen. Except for the Testis zinc finger (Testis z.f.), showing a non-specific interaction with the unrelated bait protein LaminC, all other isolated EXO1 interaction partners showed to be bait specific.

The presence of a known EXO1 binding protein among the verified hits, namely MLH1 (Table 2), confirmed the quality and reliability of the assay. Three 14-3-3 isoforms, namely beta, epsilon and zeta, were the highest hits (Table 2), with the b- being more represented than the e- and z-isoform. According to the results provided by the X-Gal assay, the strength of the EXO1/b-isoform interaction appeared to be of the same order of the EXO1/MLH1 interaction, whereas e- and z-isoform were less potent EXO1 interacting partners (Fig.17 and Table2). Another interesting putative EXO1 interacting protein was CAF1 (chromatin assembly factor 1), but according to

the X-Gal assay, the strength of this interaction was rather weak or it could be a transient interaction partner (Fig.17 and Table2).

Interactor	Function	Hits	Growth	X-gal	Bait dep.
14-3-3B	Adaptor protein	74	+++	+++	yes
14-3-3E	Adaptor protein	5	+++	++	yes
14-3-3Z	Adaptor protein	2	+++	+	yes
MLH1	Mismatch repair	16	+++	+++	yes
GRP78	Chaperone	9	+++	+	yes
Zinc finger protein 198	Transcription factor?	1	+++	-	yes
Villin 2	Structural protein	2	+++	+	yes
CAF1A	Chromatin assembly	1	+++	+-	yes
Jun dimerization prot.	Transcription factor	1	+++	+-	yes

Table 2 Summary of the hExo1 Yeast two-hybrid screen, indicating the function of the interaction partner, the abundance of the isolated clones in the screen and the interaction strength which is reflected by the β-Galactosidase activity in the X-gal assay.

2.3. Confirming the interaction between human EXO1 and 14-3-3 and refining the interaction domain

To confirm the data obtained in the yeast two-hybrid screen by an independent technique, we performed co-immunoprecipitation experiments. Given the low abundance of EXO1 in the cell (El-Shemerly, 2005), we transiently transfected HEK-293 cells with an Omni-tagged EXO1 construct (El-Shemerly, 2005) or with Omni-tagged EXO1 deletion mutant constructs. We immunoprecipitated the expressed protein using a pan-14-3-3 antibody and detected the proteins with the same pan-14-3-3 antibody or with an Omni antibody. The data showed that Omni-EXO1 and 14-3-3 proteins could be recovered as a complex (Engels et al. 2010 and Fig.18). Moreover, does the data suggest that the interaction domain on human EXO1 falls within an area spanning amino acids 366 - 548, since interaction is lost with the smallest EXO1 deletion mutant (1-348) but it is detectable with the second smallest EXO1 deletion mutant (1-548) (Fig.18). Accordingly, in the yeast two-hybrid

assay a construct containing an N-terminal deletion (366-846) showed interaction with 14-3-3 (Fig.17).

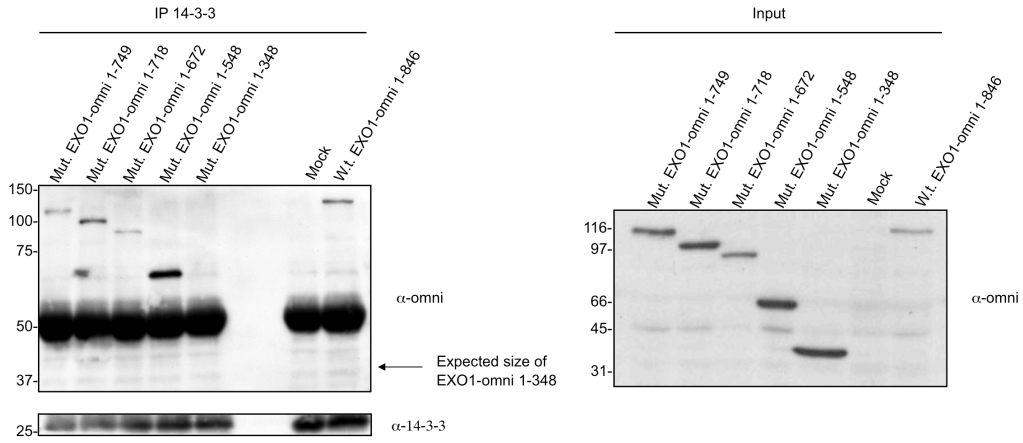


Fig.18 Co-immunoprecipitation experiment confirming the interaction data from the yeast two-hybrid screen and refining the interaction domain to a region spanning amino acids 366-548 of human EXO1

Next, we decided to assess the physiological significance of the EXO1/14-3-3 interaction. Previous studies carried out in the laboratory showed that human EXO1 is phosphorylated at nine residues (8 Ser, 1 Thr) under basal conditions and at three additional sites (2 Ser, 1 Thr) upon HU treatment. These three HU-induced sites of phosphorylation (S454, T621 and S714) conformed to the requirement for recognition by CHK1/CHK2, MAPK/SAPK and ATM/ATR, respectively (El-Shemerly et al. 2008). We therefore speculated that these sites, when phosphorylated might be required or at least facilitate binding of 14-3-3 proteins. We therefore tested the wild type EXO1, the S714 to alanine mutation and the S454, T621 and S714 to alanine triple mutation for interaction with 14-3-3 under basal conditions and upon HU treatment. The data showed that treatment with HU did not cause any variation of the interaction in the mammalian system. Neither did the single phosphoserine to alanine mutation nor the triple mutant changed the interaction strength (Fig. 19).

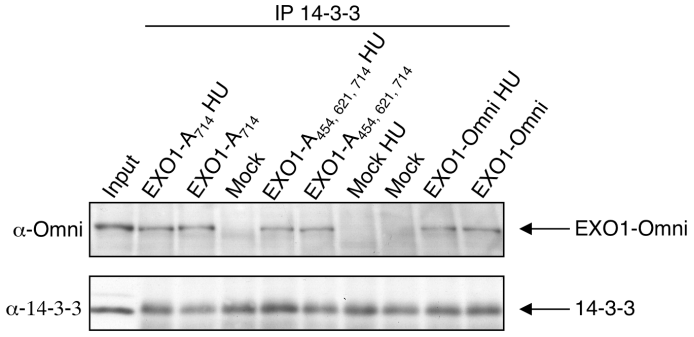


Fig.19 Co-Immunoprecipitation experiment to test if any of the HU induced phosphorylation sites on human EXO1 might influence the binding to 14-3-3 proteins

As these experiments have been carried out with overexpressed human EXO1, we cannot exclude the possibility that we have been looking at a quite artificial situation. The fact that we cannot detect any difference in interaction upon HU treatment or upon phosphoserine modifications might be due to this set up.

In order to obtain a clear-cut answer we decided to employ the yeast *Saccharomyces cerevisiae* as model organism, a system where only two 14-3-3 proteins are present, namely Bmh1 and Bmh2. In preliminary experiments we examined whether yeast Exo1 and 14-3-3 proteins interact. A C-terminal Myc- or a HA-tag was added to the endogenous *EXO1* or *BMH1/BMH2* genes, respectively. Immunoprecipitation experiments showed that Exo1 formed complexes with Bmh1 or Bmh2 in a HU-dependent manner (Engels et al. 2010).

Taken together, these data suggest that the EXO1/14-3-3 interaction is conserved from yeast to mammalian cells. Moreover, the observation that such interaction is HU-dependent in yeast, suggests that it might have a functional role during DNA replication or in response to other DNA damages.

2.4. Sensitivity to genotoxic agents

Using *Saccharomyces cerevisiae* as model organism we had the possibility to test the contributions of the proteins identified in our two-hybrid

screen to the resistance against a set of genotoxic agents, by examining defined genetic backgrounds. We tested the sensitivity of different combinations of genetic backgrounds to Doxorubicin, which is commonly used as cancer therapeutic under the name of Adriamycin, and to UV light. Adriamycin is a DNA intercalating agent that is thought to interfere with topoisomerase II, thus leading to a replication block. As expected, strains carrying a mutation in 14-3-3 were slightly more sensitive to low doses of Adriamycin when compared to wild type cells (Fig.20). On the other hand, low doses of UV radiation did not lead to a significant increase in sensitivity in the different genetic backgrounds (Fig.20).

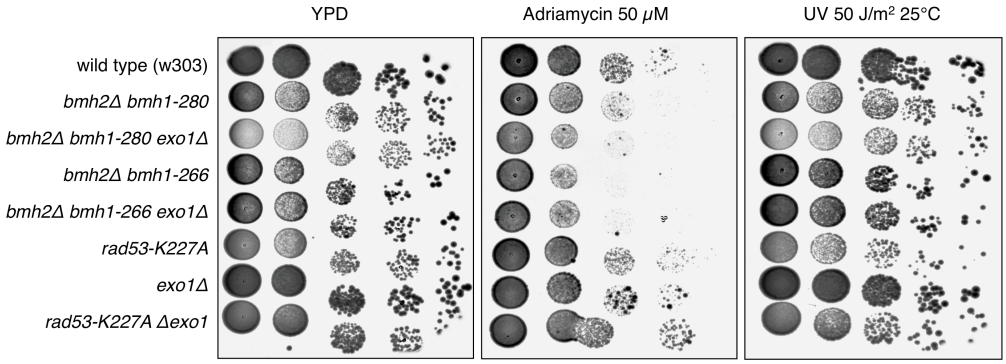


Fig.20 Adriamycin and UV-sensitivity assay. Wild type, *bmh1-280 bmh2Δ, bmh1-280 bmh2Δ exo1Δ, bmh1-266 bmh2Δ, bmh1-266 bmh2Δ exo1 Δ, rad53-K227A, exo1Δ* and *rad53-K227A exo1Δ* cultures were grown exponentially. Serial dilutions (1:10) were spotted on YPD plates containing the indicated amount of Adriamycin or spotted on YPD plates that were irradiated with the indicated dose of UV light, and grown for 3 days before scoring.

Next we went on to test the sensitivity of these strains to MMS (methyl methanesulfonate), an alkylating agent that is currently used in cancer therapy. As expected and previously reported (Lottersberger et al, 2003), the 14-3-3 strain *bmh1-280 bmh2Δ* was very sensitive to MMS, nearly to the same extent than the rad53 strain (Fig.21). Since deletion of Exo1 can partially rescue the defects of a Rad53 strain in response to MMS (Fig.21), we were very curious to see whether knocking out Exo1 in the 14-3-3 strain would give a similar pattern. Unfortunately, we did not observe any positive or negative genetic interaction between 14-3-3 and Exo1 upon MMS treatment (Fig.21).

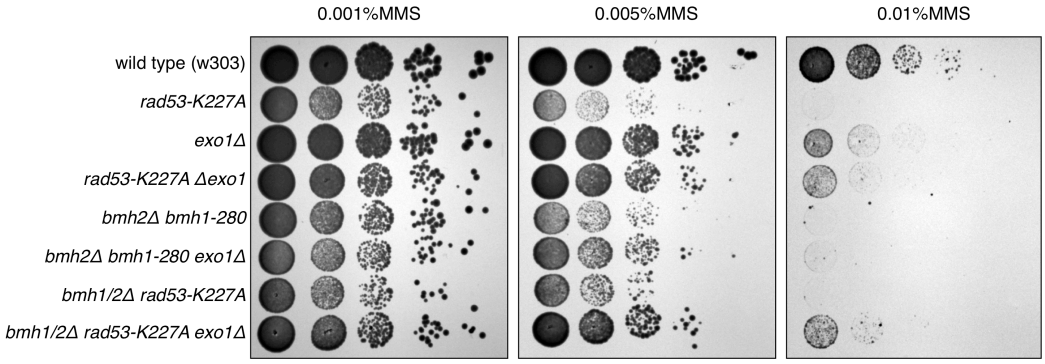


Fig.21 MMS sensitivity assay. Wild type, *rad53-K227A, exo1Δ, rad53-K227A exo1Δ, bmh1-280 bmh2Δ*, *bmh1-280 bmh2Δ exo1Δ*, *bmh1-280 bmh2Δ rad53-K227A* and *bmh1-280 bmh2Δ rad53-K227A exo1Δ* cultures were grown exponentially. Serial dilutions (1:10) were spotted on YPD plates and grown for 3 days before scoring.

Finally, the response that by far was the most interesting to us concerned the effect of HU, which is also used for several medical applications. We reasoned that HU is well suited to study the effect of gene deletion on DNA replication since it causes fork stalling by depleting the nucleotide pool. The 14-3-3 strain *bmh1-280 bmh2Δ* appeared to be very sensitive to HU treatment, virtually to the same extent displayed by the rad53 strain (Fig.22). Analogously to the MMS treatment, Exo1 could partially rescue the defects of a Rad53 strain in response to HU (Fig.22). Also in this case, we did not observe any positive or negative genetic interaction between 14-3-3 and Exo1 upon HU treatment (Fig.22).

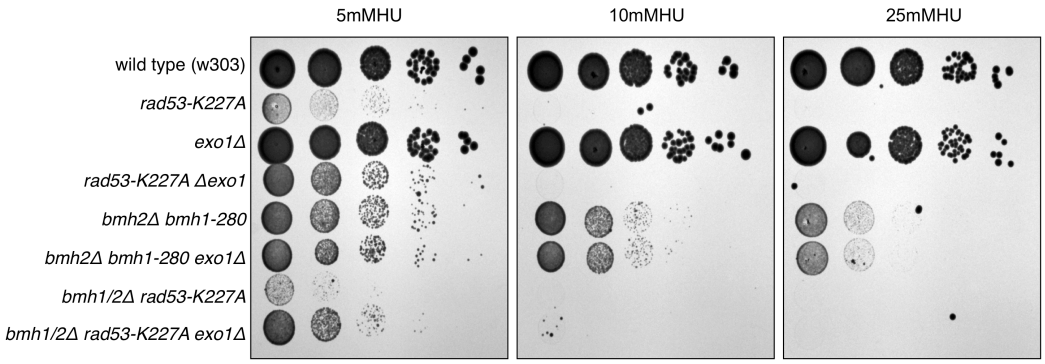


Fig.22 HU sensitivity assay. Wild type, *rad53-K227A*, *exo1Δ*, *rad53-K227A exo1Δ*, *bmh1-280 bmh2Δ*, *bmh1-280 bmh2Δ exo1Δ*, *bmh1-280 bmh2Δ rad53-K227A* and *bmh1-280 bmh2Δ rad53-K227A exo1Δ* cultures were grown exponentially. Serial dilutions (1:10) were spotted on YPD plates and grown for 3 days before scoring.

Although we did not find any direct genetic link between 14-3-3 and Exo1, the results led us speculate that a more direct visualization of the replication intermediates could provide a better insight into the events occurring at the replication fork as well as into the role of these two proteins in the control of replication.

2.5. Characterization of the replication fork progression and stability in HU

Considering that yeast 14-3-3's, Bmh1 and Bmh2 interact with Exo1 (Engels et al. 2010), we asked whether this complex would affect stability and progression of stalled replication forks. To this end, cells of the above mentioned strains, synchronized in G1 by alpha-factor treatment, were released into medium containing HU and replication intermediates (RIs) were examined by neutral-neutral 2D gel electrophoresis (2D gels) (Friedman and Brewer Meth. in Enzymol. 1995).

The probes that were designed to visualize replication fork progression in a region of Chromosome III that contains an early S-phase firing origin (ARS305) (Newlon et al., 1993), three contiguous passively replicated regions (Part A, B and C) and a further region including the dormant origin ARS301

(Part D) (Lopes et al. 2001), are shown in (Fig.23). The lower panel of the figure provides a schematic representation of the typical structures that can be visualized by means of 2D gels (Fig.23).

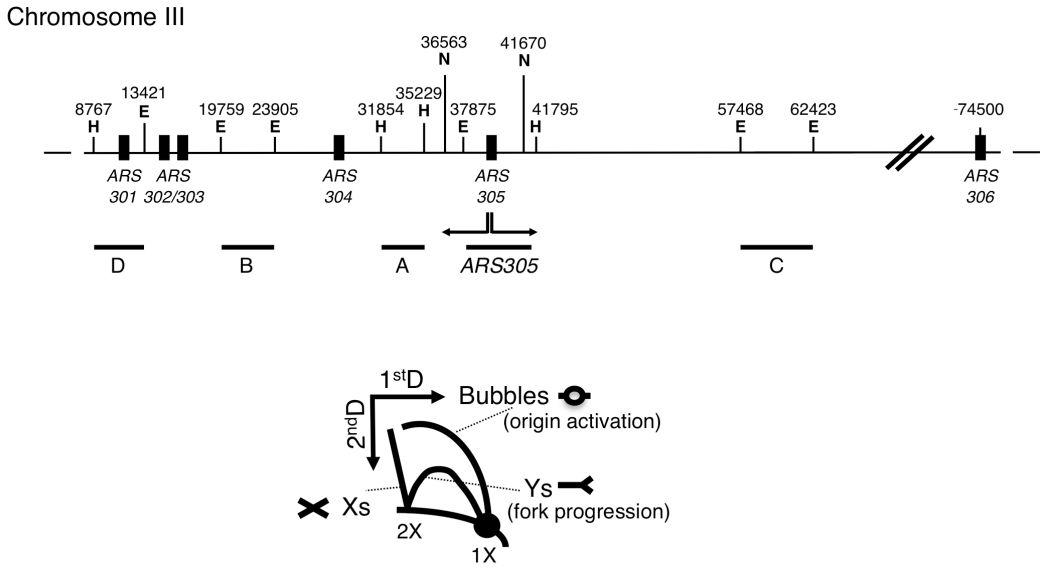


Fig.23 Schematic representation of Chromosome III and of the replication intermediates that can be visualized with the employed technique

As compared to wild-type (Fig.24), *bmh1bmh2* cells showed the same kinetics of origin firing, albeit with slightly lower efficiency as revealed by the intensity of the bubble arc at 30 min (Fig.25). Progression of the replication forks in HU from ARS305 across the region of Part A (positioned 5 Kb to the left of ARS305) was completed after 2-3h in wild-type cells, with the peak of intermediates detectable after ~1h. In *bmh1bmh2* cells the first intermediates appeared on this region with 30 min delay, whereas the peak of intermediates was delayed of ~2h as compared to wild-type cells. This pattern is indicative of a significant decrease in the rate of the replication fork progression in HU (Fig.24 and Fig.25 and Engels et al. 2010).

Since it was previously shown that yeast 14-3-3 proteins bind to the checkpoint kinase Rad53 (Usui and Petrini, 2007), we asked whether the slow fork progression in *bmh1bmh2* cells might be solely due to checkpoint defects. To address this issue, we compared Rad53-mutant cells to *bmh1bmh2* cells. Both the destabilization of replication intermediates (Fig 27 ARS305 and Part A) and the uncontrolled firing of dormant origins displayed by *rad53-K227A* cells (Fig.26 Part D) were absent in *bmh1bmh2* cells (Fig.25). As previously

reported, deletion of *EXO1* in the checkpoint defective background led to a rescue of the striking *RAD53* phenotype to almost wild type situation (Cotta-Ramusino et al., 2005) (Fig.26, Fig.27 and Fig.24), or to the phenotype of the *EXO1* deletion alone, which looks indistinguishable from wild type (Fig.27, Fig.28 and Fig.24).

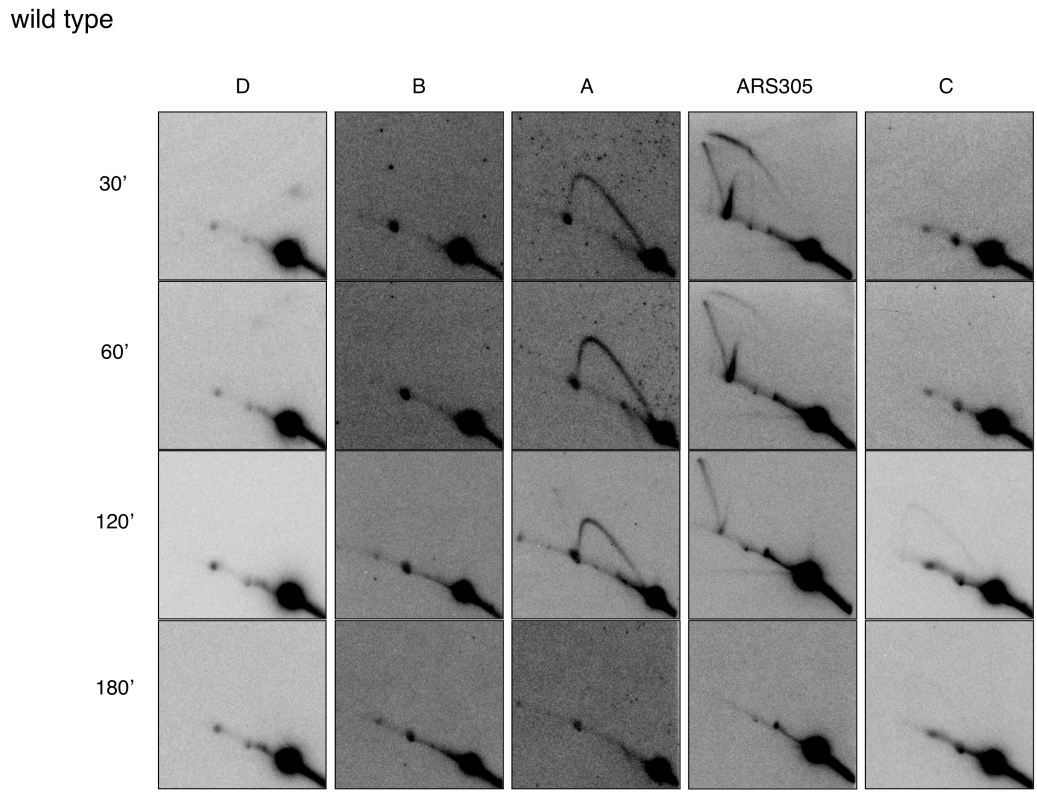


Fig.24 Time-course resolution by 2D gel analysis of replication intermediates obtained from wild type cells grown in the presence of 0.2 M HU after release from G1.

bmh1-280 bmh2Δ

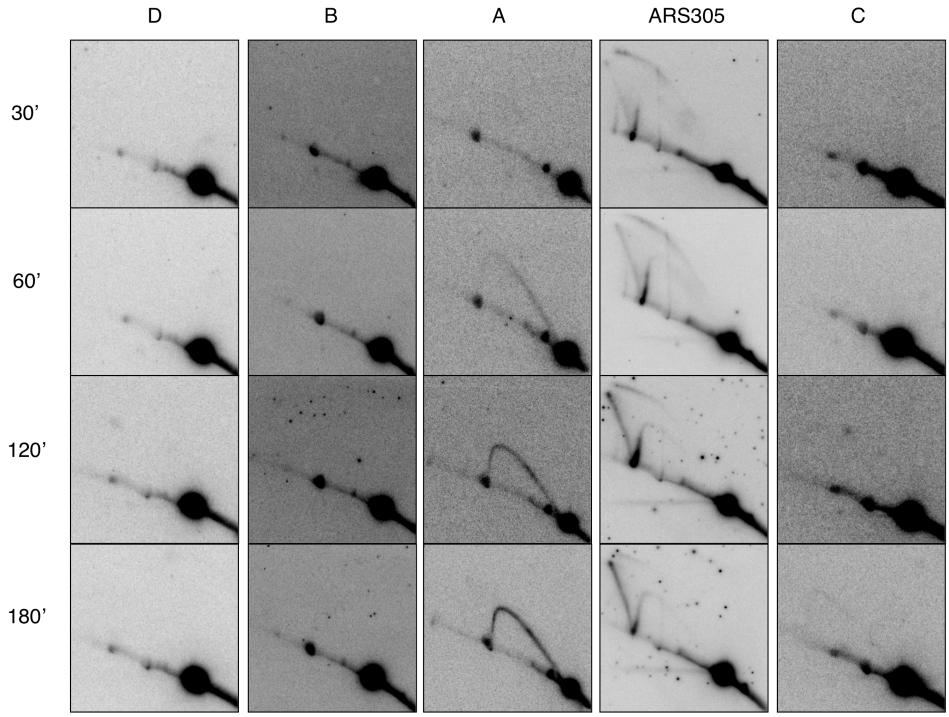


Fig.25 Time-course resolution by 2D gel analysis of replication intermediates obtained from *bmh1-280 bmh2Δ* strain grown in the presence of 0.2 M HU after release from G1.

rad53-K227A

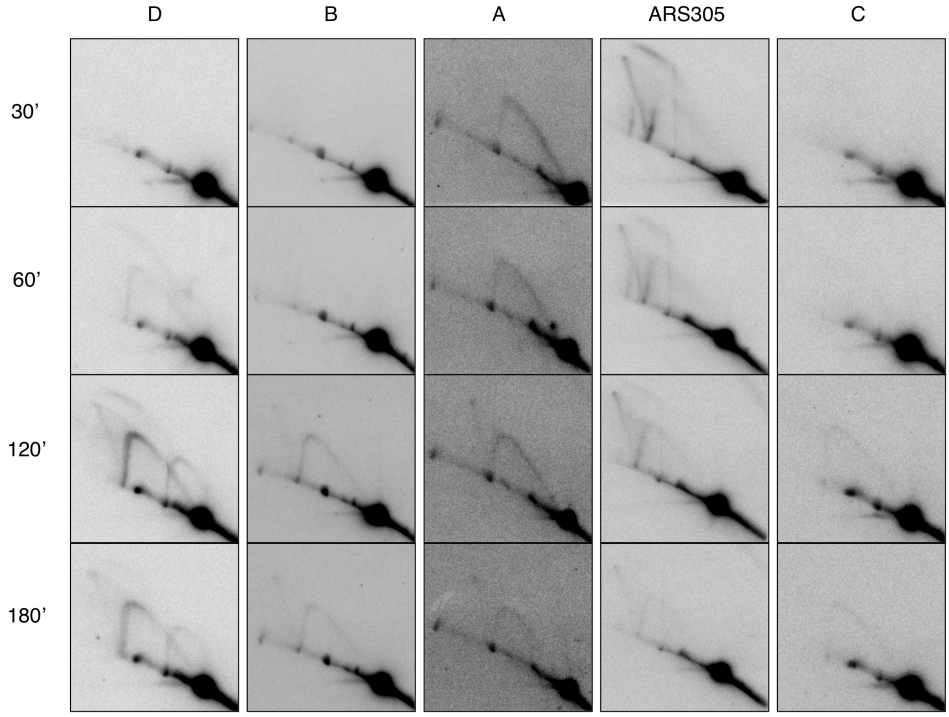


Fig.26 Time-course resolution by 2D gel analysis of replication intermediates obtained from *rad53K227A* strain grown in the presence of 0.2 M HU after release from G1.

rad53-K227A exo1Δ

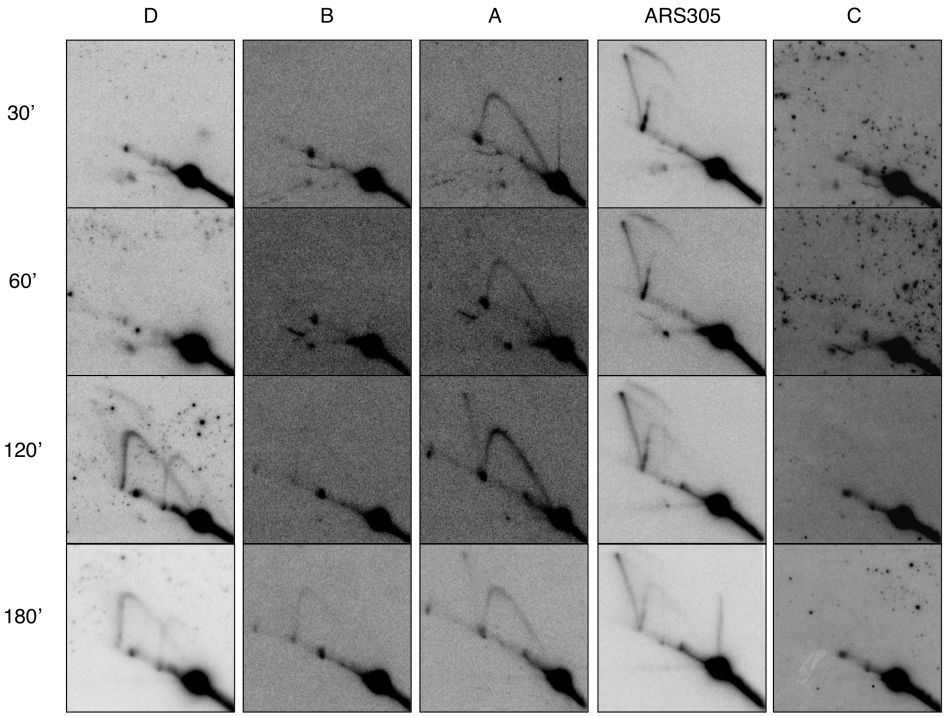


Fig.27 Time-course resolution by 2D gel analysis of replication intermediates obtained from *rad53K227A exo1Δ* strain grown in the presence of 0.2 M HU after release from G1.

exo1Δ

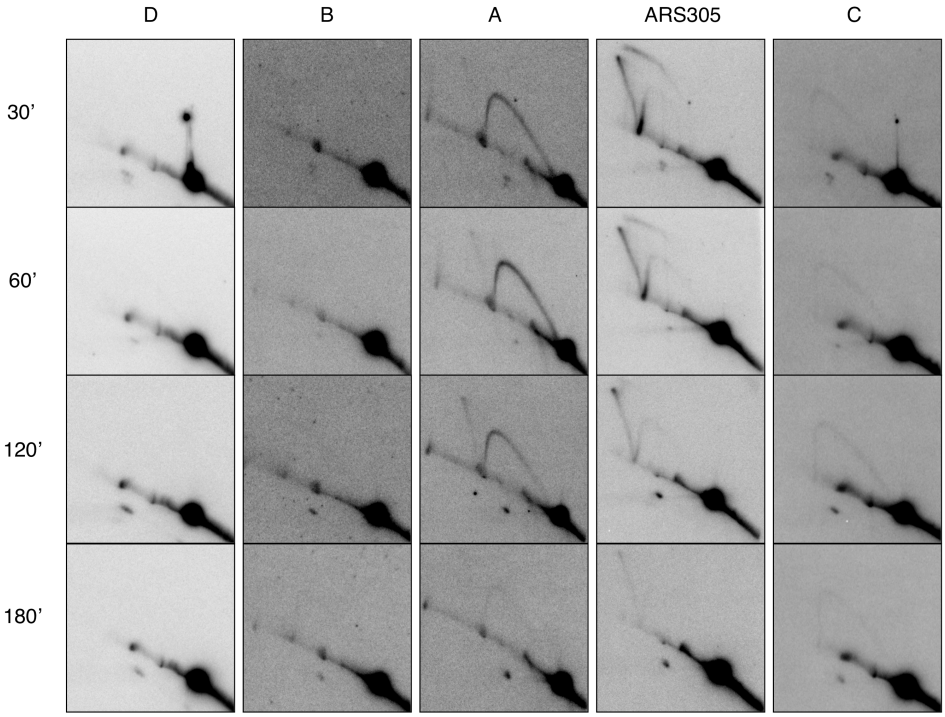


Fig.28 Time-course resolution by 2D gel analysis of replication intermediates obtained from *exo1Δ* strain grown in the presence of 0.2 M HU after release from G1.

Since it is known that *EXO1* deletion has a tremendous effect on replication fork stability in checkpoint defective cells (Cotta-Ramusino et al. 2005) and that Exo1 interacts with 14-3-3 proteins, we speculated that deletion of *EXO1* might also rescue the phenotype of 14-3-3 deficient cells. However, we observed that deletion of *EXO1* in a 14-3-3 deficient background did not rescue the slow fork progression phenotype (Fig.24 and Fig.29).

Combined deficiency of Rad53 and 14-3-3 proteins had a synergistic effect on the destabilization of replication intermediates, arguing that their effect on replication forks can be clearly dissected (compare Fig.30 with Fig.25 and Fig.26).

Deletion of Exo1 in this background led to a rescue of the destabilization of the replication intermediates but did not rescue the slow fork progression pattern, which is due to lack of 14-3-3's (Fig.31), and the firing of silent origins, which is due to lack of Rad53 (Fig.31).

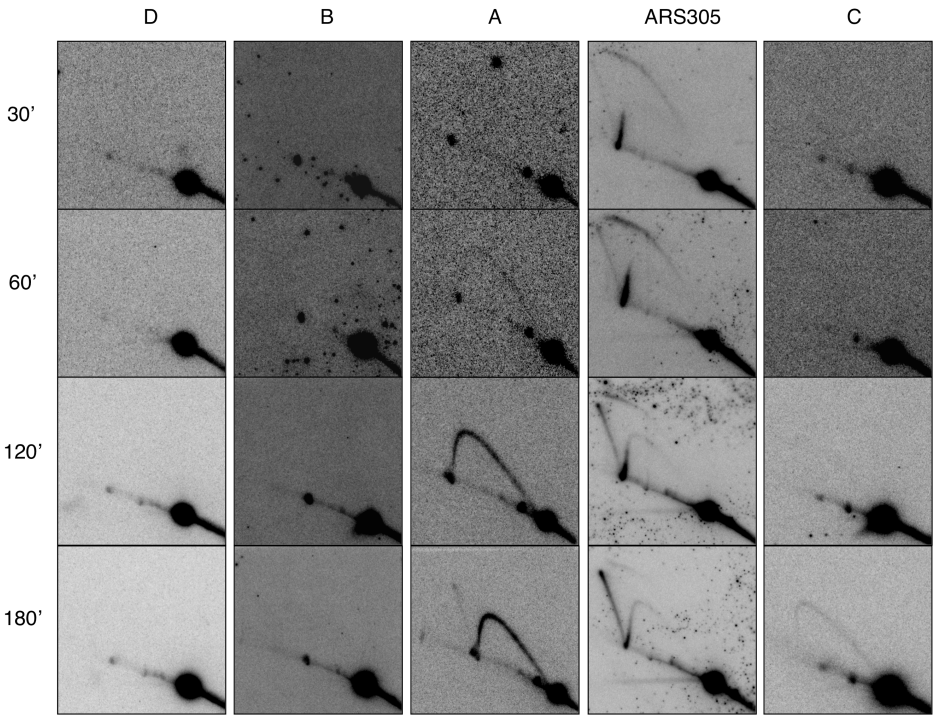


Fig.29 Time-course resolution by 2D gel analysis of replication intermediates obtained from *bmh1-280 bmh2Δ exo1Δ* strain grown in the presence of 0.2 M HU after release from G1.

bmh1-280 bmh2Δ rad53-K227A

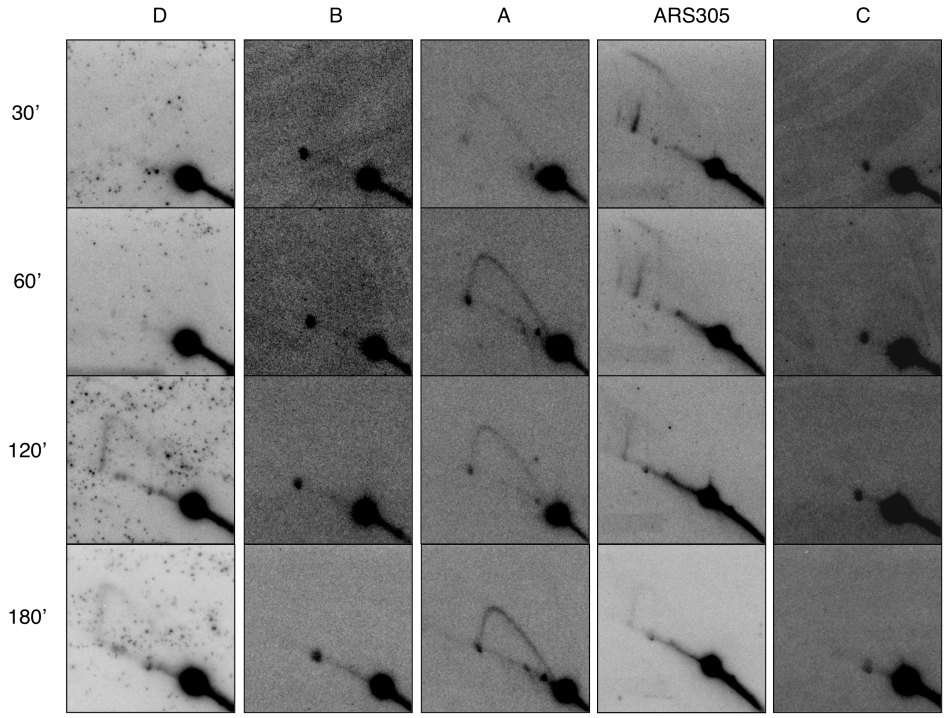


Fig.30 Time-course resolution by 2D gel analysis of replication intermediates obtained from *bmh1-280 bmh2Δrad53K227A* strain grown in the presence of 0.2 M HU after release from G1.

bmh1-280 bmh2Δ rad53-K227A exo1Δ

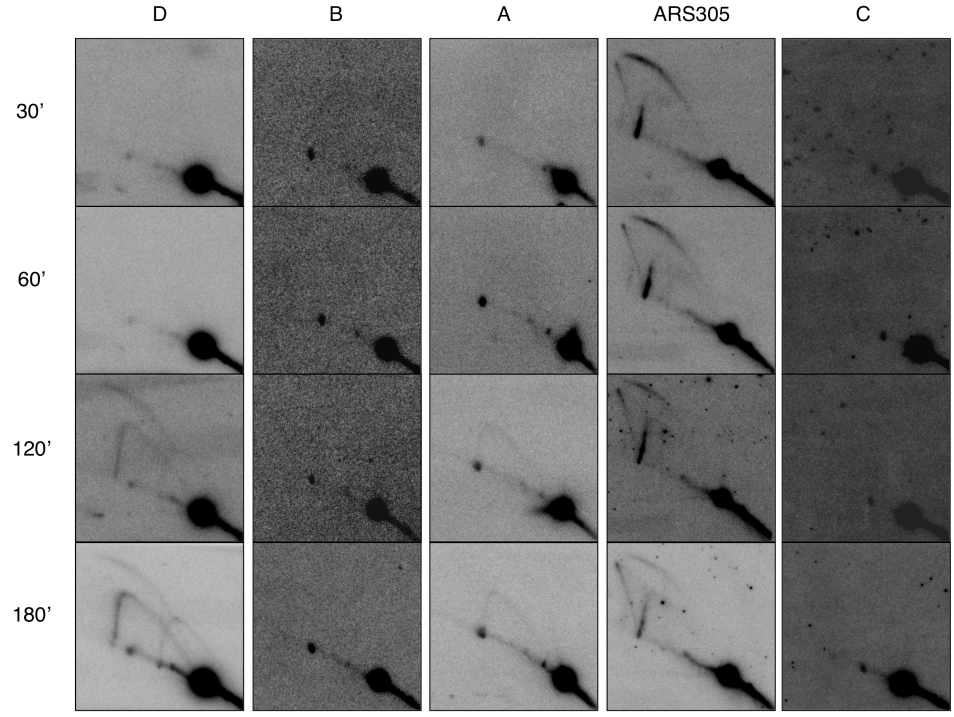


Fig.31 Time-course resolution by 2D gel analysis of replication intermediates obtained from *bmh1-280 bmh2Δ rad53K227A exo1Δ* strain grown in the presence of 0.2 M HU after release from G1.

2.6. Recovery from a HU induced replication block

Since the results presented above clearly indicate that both 14-3-3 proteins and Exo1 are involved in the dynamic events at stalled replication forks, we went on to analyze the recovery from HU induced replication fork stalling by flow cytometric analysis and 2D gel. The 2D gel uncovered that similar to checkpoint defective cells, 14-3-3 cells were also defective in resuming replication after the HU induced block. However, the replication intermediates observed in the *bmh1bmh2* strain were not destabilized and degraded as those detected in the *Rad53K227A* strain. Despite this, also such intermediates failed to resume replication (Fig.32). While *EXO1* deletion in the checkpoint defective background stabilized the replication intermediates, it did not affect the ability of these cells to resume replication. The same was observed to be the case upon deletion of *EXO1* in the 14-3-3 defective background (Fig.32).

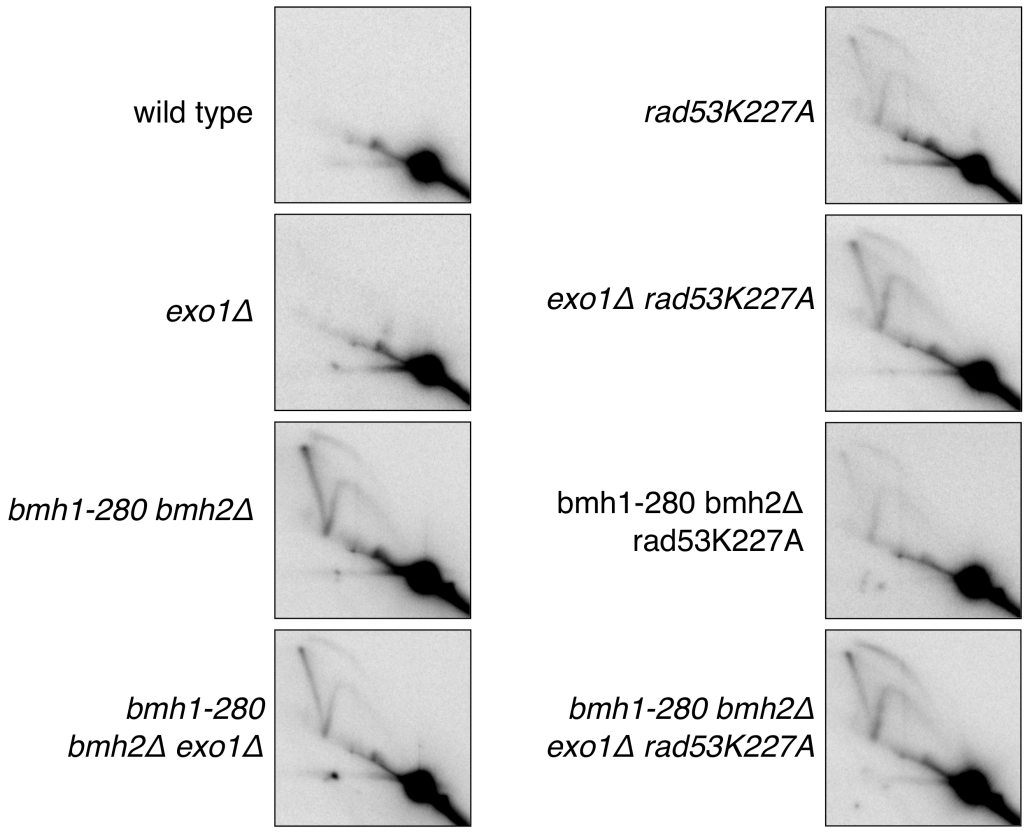


Fig.32 2D gel analysis of replication intermediates obtained from wild type, *rad53-K227A, exo1Δ, rad53-K227 exo1Δ, bmh1-280 bmh2Δ*, *bmh1-280 bmh2Δ exo1Δ*, *bmh1-280 bmh2Δ rad53-K227A, bmh1-280 bmh2Δ rad53-K227A exo1Δ* strains grown in the presence of 0.2 M HU for three hours before release into YPD for 1 hour

In order to obtain a better time course resolution of the recovery from a HU induced replication block, we decided to analyze the pattern of recovery by flow cytometric analysis. In this setting we had the possibility to analyze many time points to get a more complete picture. Flow cytometric analysis confirmed the slow recovery and revealed that deletion of *EXO1* in the *bmh1bmh2* cells led to a partial rescue of the phenotype. Such effect could be best seen at the 120 minutes time point after release from HU (Fig.33).

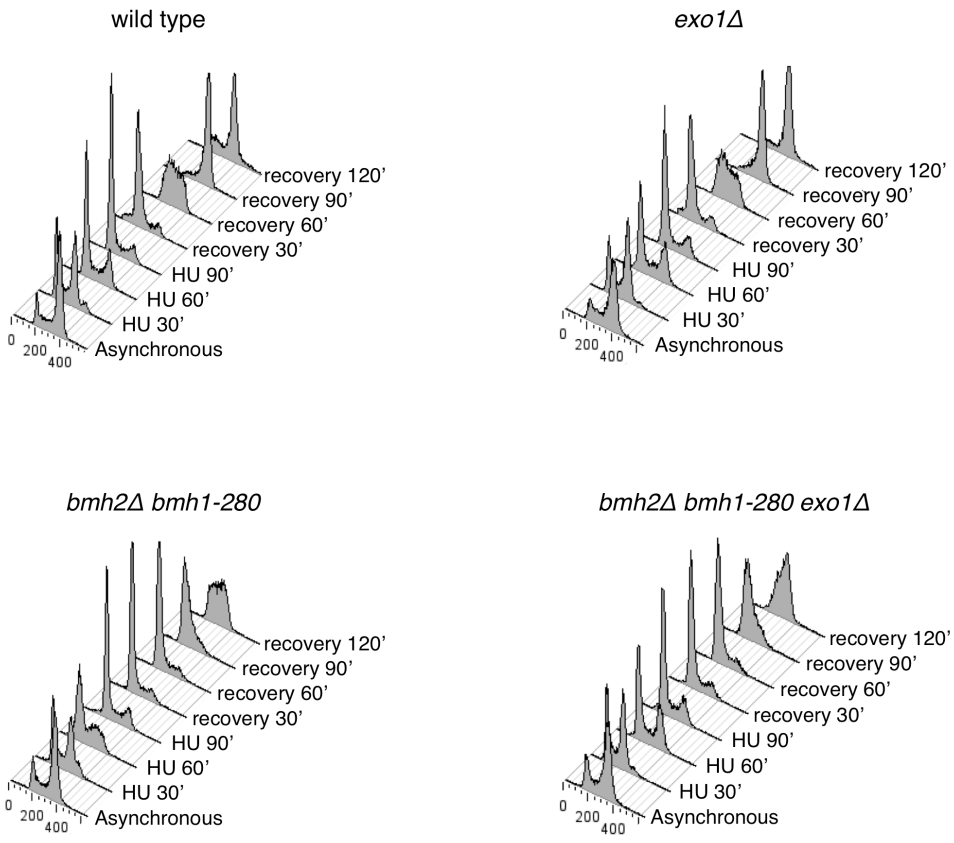


Fig.33 Time-course FACS analysis of wild type, *exo1Δ, bmh1-280 bmh2Δ*, *bmh1-280 bmh2Δ exo1Δ*, strains grown in the presence of 150 mM HU for the indicated time before release into YPD for the indicated time.

2.7. Phosphorylation of Rad53 and Exo1 regulate checkpoint and cell cycle

The above displayed evidence prompted us to ask whether *EXO1* deletion in *bmh1bmh2* cells may affect Rad53 activity. Western blot analysis showed that, compared to wild type cells, Rad53 was hyperphosphorylated in HU-treated *bmh1bmh2* cells and that its dephosphorylation was retarded during the HU-recovery phase, thus correlating with the described replication restart defect. Importantly, deletion of *EXO1* in 14-3-3-deficient cells re-established to a great extent the pattern of rapid Rad53 dephosphorylation in the recovery phase, substantiating the flow cytometry data (Engels et al. 2010).

Our lab has previously shown that upon replication perturbations, human EXO1 undergoes rapid phosphorylation that targets it for degradation to prevent

any unscheduled nucleolytic activity (El Shemerly 2005). Recently, yeast Exo1 was also shown to undergo Mec1/Rad53-mediated phosphorylation, a post-translational modification that regulates its activity at telomeres (Morin et al. 2008). This evidence prompted us to ask whether yeast Exo1 would also undergo phosphorylation upon replication fork stalling. The evidence that we present in (Engels et al. 2010) clearly shows that yeast Exo1 was phosphorylated in response to HU and that Exo1 phosphorylation was Mec1-dependent. Most importantly, we show that the high stoichiometry of Exo1 phosphorylation observed in wild type cells could not be reached in 14-3-3 deficient cells and additionally, that the rate of Exo1 dephosphorylation upon recovery from HU was considerably reduced in 14-3-3 deficient cells (Engels et al. 2010).

2.8. Exo1 dependent accumulation of ssDNA gaps behind the fork in *bmh1bmh2* cells

In order to get more structural insights into the events at stalled replication forks, we used electron microscopy to directly visualize the structures that arise in response to HU treatment. Therefore, we synchronized the cells in G1 and released them for 1 or 2 hours, respectively, in YPD medium containing 0.2 M HU. Cells were cross-linked *in vivo* with psoralen, enriched in replication intermediates by BND cellulose chromatography, and analyzed by electron microscopy (EM) under non-denaturing conditions (Lopes 2009). Initially, for most strains and time points, 30 replication intermediates were scored and analyzed to obtain a first glimpse of the phenotype. After screening the different time points and strains, we focused on the one-hour HU time point because the observed phenotype was most pronounced at that time. For each strain, showing an interesting phenotype, and the relevant control strains, more or less 100 replication intermediates were analyzed in duplicate. Phenotypes characteristic of checkpoint defects (Sogo et al, 2002) were not observed in 14-3-3 deficient cells: ssDNA regions directly at the elongation points were as frequent and long as in wild-type cells and no fork reversal could be observed

(data not shown). However, 14-3-3-deficient cells showed a dramatic accumulation of ssDNA gaps behind the replication fork (Engels et al. 2010). Statistical analysis indicated that approximately 50% of all replication intermediates analyzed displayed one or more ssDNA gaps (Engels et al. 2010). Interestingly, deletion of *EXO1* in *bmh1bmh2* cells completely suppressed this phenotype, leading to a reduction of the ssDNA gaps behind the fork to a level similar to wild-type or *exo1Δ* cells (Engels et al. 2010). The comparison of ssDNA gaps length scored by EM evidenced a striking difference: whereas *bmh1 bmh2* cells displayed a significant number of large size gaps (>0.5 Kb), the latter were absent in *bmh1bmh2 exo1Δ* cells (Engels et al. 2010). The findings from this and the previous section are described in more details in (Engels et al., 2010).

3. DISCUSSION

The present study describes a novel role of 14-3-3 proteins as key regulators of Exo1 function and in promoting fork progression, stability and restart in response to DNA replication perturbations. The identification of 14-3-3's as human EXO1 interaction partner by means of a yeast two-hybrid screen was the starting point for this project. The yeast two-hybrid screening approach is a very useful genetic assay when it comes to identify and characterize interactions between soluble proteins. This system was originally developed by Fields and Song (1989) to detect protein-protein interactions in a cellular setting. The system is based on the modular nature of transcription factors, which have a DNA binding domain and an activation domain. The bait protein of interest is fused to the DNA binding domain and as a prey, a library of cDNA fragments is fused to the activation domain of a transcription factor. When expressed separately in a yeast reporter strain these constructs do not lead to an activation of the reporter genes. However if bait and prey constructs are co-expressed in the same yeast cell and the bait protein of interest interacts with a prey protein, this leads to the reconstitution of a transcription factor, which will activate the yeast reporter genes and likewise enable the isolation and characterization of the interacting proteins. We first tested if we could express the full length human EXO1 fused to the DNA binding domain in the yeast reporter strain. Unfortunately the full-length construct was not expressed well or it was expressed and rapidly degraded by the yeast cells, possibly as a mechanism of self-defence from the unscheduled nuclease activity of the over expressed protein. We then decided to test a construct of human EXO1 lacking the first 365 amino acids of the protein, the catalytic domain, and found it to be well expressed in the yeast reporter strain. These findings confirm our speculation that the nucleolytic activity of the over expressed protein was not well tolerated by the yeast cells. Although we were using a deletion mutant to screen for novel interacting proteins we were quite confident that this construct would be useful since it retains the entire interaction domain of EXO1, which is responsible for virtually all the interactions that have been described so far. The screening approach led to the isolation of nine putative interaction partners of human EXO1. The presence of a strong and already known interaction partner,

namely MLH1, under the identified candidate proteins validated our screening approach and gave us confidence on the reliability of the results. Both the frequency with which certain interacting proteins are found in the screening and the intensity of the β-Galactosidase reporter activity can give a hint towards the interaction strength. According to this criterion, the most promising novel interaction partners were the 14-3-3 proteins. We found three different isoforms of the 14-3-3 proteins, namely 14-3-3-β, -ε and –ζ. Especially the β-isoform was a very abundant hit in the screening and, in addition, the observed β-Galactosidase activity in the bait dependency assay was of the same order than the one observed for MLH1, suggesting that it is indeed a very strong EXO1 interaction partner. Moreover, does the presence of the other 14-3-3 isoforms suggest that this interaction might be crucial for the control of EXO1 since it is known that the different homo- and hetero-dimers combinations formed by 14-3-3 proteins can affect their influence on target proteins. Another interesting putative interaction partner that we found in our screening is CAF1A, the Chromatin Assembly Factor 1 that is implicated in histone deposition on newly synthesized DNA and was previously linked to the mismatch repair system. However, according to the β-Galactosidase assay, the strength of this interaction is rather weak. We were also unable to confirm this interaction with an independent technique such as co-immunoprecipitation (data not shown). Since the yeast two-hybrid approach can also indicate weak and transient interactions it is possible that this interaction is not strong or enduring enough to be detected by co-immunoprecipitation where stable protein complexes are required.

Co-immunoprecipitation experiments did not only confirm the interaction between human EXO1 and 14-3-3's, but they also allowed us to narrow down the interaction domain on EXO1. Using five different deletion mutants of EXO1 for IP and combining the results with the yeast two-hybrid data we were able to narrow down the interaction domain to a region spanning amino acids 366 to 548. Previous work from our lab has shown that, upon HU treatment, human EXO1 undergoes rapid phosphorylation that targets the protein for degradation (El-Shemerly et al. 2005). Since a role in response to replication perturbations has been described for 14-3-3 proteins, we thought that the interaction strength might vary in response to HU treatment. On the

contrary, we did not observe an enhancement or a reduction of the interaction strength in response to HU treatment. Also did mutation to alanine of several of the putative 14-3-3 phosphoserine binding sites not change the pattern of interaction. Unfortunately, these experiments had to be carried out with overexpressed human EXO1, since no good commercial antibody that can detect endogenous human EXO1 is available. Given this caveat, we cannot rule out the possibility that overexpression of EXO1 might hamper our conclusions.

Employing the yeast *Saccharomyces cerevisiae* as model organism we were able to address the above questions in a more natural setup by analyzing interaction of the endogenous proteins. We found both yeast 14-3-3's, Bmh1 and Bmh2 to interact with Exo1 in an HU dependent manner, confirming our speculation that the functional relevance of this interaction might be linked to events at challenged replication forks. It is conceivable that the HU dependent binding in yeast, and the HU independent binding in human cells, might reflect the different modes of regulation in mammalian versus yeast cells. In contrast to mammalian cells, yeast Exo1 does not undergo degradation upon HU treatment, but phosphorylation restrains its nuclease activity (Morin et al. 2008).

14-3-3 proteins have been implicated in the hypersensitivity to genotoxic stress caused by agents such as MMS or HU (Lottersberger et al. 2003). Since a complete knockout of both isoforms is not viable we employed the mutants generated for the authors of the above-mentioned publication. In this set up, both *BMH1* and *BMH2*, the major and the minor 14-3-3 isoform, respectively, are deleted and *BMH1* is complemented by mutant *BMH1* alleles that cause several defects due to reduction of the functional level of 14-3-3 proteins but confer viability to the cells. We employed two different 14-3-3 mutant alleles, of which one is a temperature sensitive allele *bmh1-266 bmh2Δ*, bearing three point mutations. This allele allows to completely switch off the 14-3-3 function by shifting the cells to the non-permissive temperature of 37°C. The other mutant allele that we subsequently used for most studies is *bmh1-280 bmh2Δ*, carrying a single point mutation that renders the cells hypersensitive to HU and MMS, two agents that interfere with DNA replication. In order to assess genetic interactions, we deleted *EXO1* in 14-3-3 deficient cells and additionally combined them with a Rad53 mutant allele *rad53K227A*, which in

contrast to a complete knock out, allows the cells to survive because of the residual checkpoint kinase activity. These strains were employed to assess the individual genetic contributions to the resistance against various genotoxic agents. As reported previously, we observed that the *bmh1-280 bmh2Δ* and *rad53K227A* strains were sensitive to MMS and HU treatment and deletion of *EXO1* could suppress the sensitivity of the checkpoint defective *rad53K227A* allele towards both agents. The rescue of the viability in response to MMS was reported previously (Segurado and Diffley, 2008). On the contrary, Segurado and Diffley did not observe the EXO1 deletion-dependent rescue to HU treatment that we report in our study. This is very likely due to the conditions used: whereas these authors used HU at 20 mM, a dose at which both strains are unviable and therefore one cannot observe a rescue, we used a very low dose of 5mM. Unfortunately we did not observe any genetic interaction between *EXO1* and *BMH1* or *BMH2* in response to the employed genotoxic agents. The *bmh1-280 bmh2Δ rad53K227A* strain showed a slightly enhanced sensitivity towards HU treatment when compared to the individual mutants. This finding supports the idea of a synergistic effect between 14-3-3's and Rad53 proteins in response to replication fork stalling. This induces to further argue that both proteins are not only working in the same pathway, where 14-3-3's physically bind and stabilize an active conformation of Rad53 (Usui and Petrini, 2007), but additionally point to a secondary pathway by which 14-3-3 proteins alone might contribute to the stability or progression of replication forks in response to HU treatment.

By means of 2D gel electrophoresis we were able to show that 14-3-3 proteins are required to sustain the rate of DNA synthesis under low nucleotide conditions. In 14-3-3 deficient cells, fork progression is markedly reduced, to an extend comparable to the *rad53 K227A* cells with the striking difference that processing and collapse of the slow progressing forks and late origin firing does not occur. This is a very important piece of evidence, since the data on physical and functional interaction of 14-3-3 proteins and Rad53 might have been taken as suggestion of an indirect effect of 14-3-3 proteins on fork progression: our data now allow to definitely rule out this possibility.

Compared to *wild type* cells the only difference that we observed in 14-3-3 deficient cells was slow fork progression from early firing origins. These findings are very interesting considering that Mec1 and Rad53 prevent late origin firing, stabilize stalled replication intermediates and block entry into mitosis. However, the fourth cellular response to disruption of DNA replication, namely slowing of the elongation, so far could not be attributed to any of these kinases or to any other factor. Although it was previously shown that 14-3-3 proteins bind origins of replication and cruciform DNA (Alvarez et al., 2002), we cannot fully support such argument, which claims that the slow cell cycle progression observed in 14-3-3 deficient cells is solely due to defective origin firing. Although we observe a slight reduction of the kinetics of origin firing by 2D gel, the cell cycle defect cannot be attributed to this event alone. In the latest publication from this laboratory the authors claim that the slow cell cycle progression in 14-3-3 deficient cells might also be related to defects during the elongation steps. Compelling evidence for this argument is, however, not provided in the study (Yahyaoui and Zannis-Hadjopoulos, 2009).

The slow fork restart that we observe in 14-3-3 deficient cells upon HU removal, points to a recovery defect without evident fork collapse. The large majority of recovery defects occur when fork architecture is severely compromised. Our data, however, indicate that misregulation of the replisome, without dramatic physical processing of the forks, might be sufficient to prevent fork restart. We obtained the first hint pointing at a regulatory role of 14-3-3 proteins on Exo1 at replication forks, when we performed a time-course flow cytometric analysis. Upon recovery from a HU induced replication block, deletion of *EXO1* in a *bmh1-280 bmh2Δ* strain led to a slightly faster recovery. This rescue upon *EXO1* deletion in *bmh1-280 bmh2Δ* cells is also confirmed by a faster Rad53 inactivation upon HU removal. Finally, the improved resolution of Exo1 phospho-forms obtained in our study, in comparison to previously published data, led us conclude that the Mec1-dependent Exo1 hyperphosphorylation in response to replicative stress is mediated by 14-3-3 proteins. The observation that in *bmh1-280 bmh2Δ* cells, Rad53, which is another Mec1-dependent checkpoint target, was promptly phosphorylated, supports the idea that defective Exo1 phosphorylation in absence of 14-3-3

proteins is not an indirect consequence of slower fork progression and/or defective checkpoint activation. Since phosphorylation restrains yeast Exo1 activity at telomeres (Morin et al. 2008), we propose that this may also be the case at stalled forks of replication and that 14-3-3 proteins may act as a platform to mediate phosphorylation events at such structures. Electron microscopy provided direct, *in vivo* evidence for uncontrolled Exo1 activity in HU-treated 14-3-3 deficient cells. The accumulation of long ssDNA gaps behind the slow moving replication forks in *bmh1-280 bmh2Δ* cells, was completely suppressed by *EXO1* deletion.

These findings are of high significance, since they suggest that a loose control of Exo1 activity may render DNA synthesis more discontinuous in conditions of replicative stress. The stalling of the polymerase due to low nucleotide levels might lead to increased repriming events, thus raising the number of accessible 5'-ends, available for processing by Exo1. It is conceivable that in this setting, a strict control of Exo1 activity is required to limit damage. The resolution limit of electron microscopy might have prevented the detection of these nicks/small gaps in this as well as in previous studies (Sogo et al. 2002). However, in 14-3-3 deficient cells where control of Exo1 activity by phosphorylation is impaired, the unleashed exonucleolytic activity would enlarge gaps beyond the detection limit.

The fact that *EXO1* deletion suppresses the accumulation of ssDNA gaps in 14-3-3 deficient cells but not the HU sensitivity or only partially the recovery defect upon HU removal, suggests that 14-3-3 proteins might regulate additional targets (depicted as factor X in Fig.34) by modulating their phosphorylation, during replication stress (Fig.34).

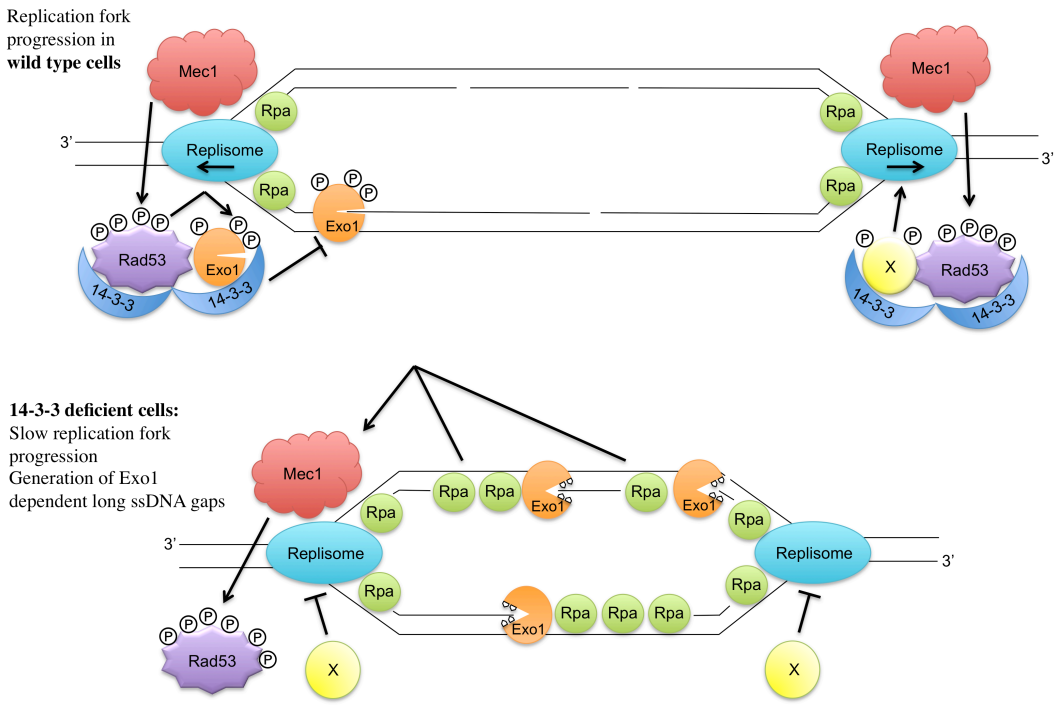


Fig.34 Hypothetical model showing the regulation of different targets by 14-3-3 proteins

Given the role of 14-3-3 proteins as integrators of signaling pathways (Morrison, 2009) and the multiplicity of their targets (Jin et al. 2004), 14-3-3 proteins might function as central regulators of the checkpoint response. Considering previously reported cases (Braselmann and McCormick) and the dynamic nature of 14-3-3 dimers (Yang et al. 2006), one may envisage a role for 14-3-3 proteins as docking clamp tethering Exo1 and the kinase controlling its activity. The most promising kinase candidate would be the Rad53 kinase, as it was previously shown to interact with 14-3-3 proteins (Usui and Petrini, 2007) and it is required for Exo1 phosphorylation (Morin et al., 2008).

4. CONCLUSIONS AND PERSPECTIVES

We can conclude from our results, that 14-3-3 proteins are *in vivo* interaction partners of Exo1, both in yeast and mammalian cells. Yeast 14-3-3-deficient cells fail to induce Mec1-dependent Exo1 hyperphosphorylation upon replication fork stalling and therefore accumulate Exo1-dependent ssDNA gaps at stalled forks. Accumulation of ssDNA gaps causes persistent checkpoint activation in these cells and contributes to the exacerbated recovery defects. Moreover, we can conclude that 14-3-3 proteins promote the rate of fork progression under limiting nucleotide concentrations.

This work clarifies events occurring at stalled replication forks, and proposes 14-3-3 proteins as central integrators of signals that regulate fork stability, progression and processing. 14-3-3 proteins regulate the phosphorylation of Exo1 and other unknown targets and likewise control their activity (Fig.34).

Challenges lying ahead consist in the identification of components of the replisome, or proteins controlling them, that may be 14-3-3 targets, as well as in the elucidation of the exact mechanism by which 14-3-3 modulate Exo1 activity (Fig.34). Additionally, it would be of great interest to confirm in human cells the data obtained in the yeast model system.

5. MATERIALS AND METHODS

Materials

The antibodies used in this study were: mouse monoclonal anti-LexA (sc-7544, Santa Cruz Biotechnology); goat polyclonal Omni-probe (M21, sc-499, Santa Cruz Biotechnology); rabbit polyclonal anti-pan 14-3-3 (SA-483, Biomol); mouse monoclonal anti-HA (12CA5, Sigma) and anti-Myc (9E10, Santa Cruz Biotechnology); rabbit polyclonal anti-Rad53 (a kind gift from C. Santocanale, Galway, Ireland).

The chemicals and peptides used in this study were: Hydroxyurea (Sigma); Methyl methanesulfonate (Sigma); Adriamycin (Calbiochem); X-gal (Qbiogene); a1-Mating Factor (Sigma).

Saccharomyces cerevisiae strains

The yeast strains used in this study are isogenic to W303-1A (wild-type) (Thomas and Rothstein, 1989), CY2034 (*rad53-K227A*), CY5145 (*exo1Δ*), CY5469 (*rad53-K227A exo1Δ*), KE2 (*bmh2Δ bmh1Δ::bmh1-280*), KE3 (*bmh2Δ bmh1Δ::bmh1-266*), KE4 (*bmh2Δ bmh1Δ::bmh1-280 exo1Δ*), KE5 (*bmh2Δ bmh1Δ::bmh1-266 exo1Δ*), KE7 (*bmh2Δ bmh1Δ::bmh1-280 rad53 Δ::rad53-K227A)*, KE8 (*bmh2Δ bmh1Δ::bmh1-280 rad53 Δ::rad53-K227A exo1Δ),* KE15 (*bmh1-3HA exo1-9myc*), KE16 (*bmh2-3HA exo1-9myc*), THY AP4 (*ura3, leu2, lexA::lacZ::trp1, lexA::HIS3, lexA::ADE2*), YMG1009 (*exo1-9myc*), YMG1197 (*bmh2Δ bmh1Δ::bmh1-280 exo1-9myc*), YLL909 (*bmh1-3HA*), YLL910 (*bmh2-3HA*), YMG1201 (*exo1-9myc-HIS3*), YMG1215 (*mec1Δ sml1Δ exo1-9myc-HIS3*).

Yeast two-hybrid screen

The yeast two-hybrid screening was performed with DN-EXO1 ($EXO1_{366-846}$) as bait on a cDNA library generated from human peripheral blood

mRNA (a kind gift of I. Stagljar, Toronto, Canada) as described previously (Jiao R. et al., 2004) and using THY AP4 as reporter strain.

Cell culture

HEK-293 cells were maintained and transiently transfected as described (El-Shemerly et al, 2005).

Protein extraction, Western Blotting, Immunoprecipitaion

Human cellular proteins were extracted using ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-PPi, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40). Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad). Omni-EXO1 or 14-3-3 proteins were immunoprecipitated from 2 mg total HEK-293 cell extracts for 3h at 4°C using either the Omni-probe antibody or the pan-14-3-3 antibody. Antibodies were captured using protein G-agarose beads (Santa Cruz Biotechnology) for 1h at 4°C. Beads were washed in 2x 1 ml TNET ice-cold buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100) followed by 2x 1 ml ice-cold TNE buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM EDTA) and heated for 10 min at 95°C in 2 x Laemmli sample buffer. Proteins were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) (GE-Healthcare) and the membrane was probed with appropriate antibodies. Immune complexes were revealed using the enhanced chemiluminescence system (GE-Healthcare).

Yeast cellular proteins were extracted using ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 15 mM NaCl, 15 mM EGTA, 1mM NaF, 1mM Na orthovanadate, 4 mM p-Nitro-Phenyl-Phosphate (pNPP), 0.1% Triton X-100, 1mM PMSF, complete protease inhibitors cocktail (Roche)). Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad). 14-3-3-HA was immunoprecipitated from 10 mg total cell extracts for 2h using the

monoclonal HA antibody. The antibody was captured for 1h at 4°C using protein G-agarose beads. Beads were washed in 4 x 1ml ice-cold lysis buffer and heated for 10 min at 95°C in 2 x Laemmli sample buffer. Proteins were resolved on 7.5% or 10% SDS-polyacrylamide gels and detected as described above using anti-HA or anti-Myc monoclonal antibodies.

For Western blot experiments, yeast cellular proteins were extracted using the TCA method (Muzi-Falconi M. et al., 1993), resolved on 10% SDS-polyacrylamide gels and detected with a rabbit polyclonal anti-Rad53 antibody.

For the visualization of Exo1 phosphorylation, an optimized Phos-tag system (5 mM Phos-tag reagent) was employed according to (Kinoshita E. et al., 2008). Proteins were transferred to nitrocellulose (porablot NCP, 0.45 μm pore size, Machery-Nagel) overnight at room temperature applying constant amperage (200 mA) and detected as described above.

Flow cytometric analysis

Flow cytometric analysis was performed as described (Pellicioli, A. et al., 1999).

2D Gel Electrophoresis and Electron Microscopy

DNA extraction with the CTAB method and neutral-neutral two-dimensional gel electrophoresis were performed as described (Lopes at al. 2003). Replication intermediates quantification (Lopes et al., 2001) and EM analysis (Lopes M., 2009) were performed as described.

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